

- **Biotech Cluster**

- Using statistics to fine-tune a product**

During the Biomedical Tuesday meeting in September, **Adrian Rubio**, lab manager of **Theranostech Inc.**, and **Bert Davis**, statistical consultant, talked about a study Theranostech conducted that harnessed statistical analysis to improve the product.

Theranostech, founded in 2004, has two patents and two patents pending, Adrian told members of the New Mexico Biotechnology & Biomedical Association. The primary product is the Tagger HIV-1, a kit, which contains an antibody cocktail, a corresponding isotype control cocktail, a suspension of gp120-labeled positive-control beads, a concentrated wash buffer and a phosphate buffered saline solution. The antibody cocktail is a mixture of four monoclonal antibodies to gp120, a protein on the HIV surface that initiates the viral invasion of the cell, and one antibody to CD4. The antibody cocktail is able to detect multiple mutations of HIV.

"CD4 cells are the crux of a lot of immunological activity in the body," Adrian said.

Their pre-clinical data show that the Tagger HIV-1 detects and measures the degree of the infection in patients by identifying the number of infected cells.

Bert explained that he used a box-whisker plot to look at the body of data. It demonstrated that the normal population is very homogeneous while the infected population is quite heterogeneous.

Later, he employed a radar-operating characteristic curve to compare different diagnostic techniques. The ROC curve is a technique that originated in the radar community, but the medical community picked it up for comparing diagnostic methods. He used linear discriminant analysis to combine the data from the four quadrants to predict if an individual sample came from the normal or the infected population.

"The analysis provides a huge jump in the ability of the technique to find the virus when much of it is below the detection point for traditional methods," Bert said.

His analyses showed the Tagger could differentiate between HIV negative and HIV positive blood specimens. And it could detect HIV positive lymphocytes in one sample when viral RNA levels were below the Roche Amplicor detection limits.

COO **Jess Stengel** explained that the cause of the disease was discovered in 1983, but it took researchers a long time to understand how it operates. Currently the conventional screen is the Roche test, which measures viral nucleic acid for HIV positive individuals during tests four times a year. But it takes the Roche test about two weeks to produce a result. Theranostech is working hard to provide results in less than a week, Jess said.

"Our test monitors more pathologically relevant measures — the infected cell," he said. "We're now in the process of taking Bert's work and tweaking the product to establish clinical relevance to a greater degree."



# Theranostech, Inc.

January 25, 2005

## Executive Summary

Theranostech, Inc. is a late development-stage biotechnology company incorporated in April 2004, as a privately held New Mexico "C" Corporation. Theranostech's core business is the development, manufacturing and marketing of theranostic biotechnology products for the diagnosis and monitoring of infectious diseases.

Theranostech has four full-time and one part-time employees and a team of consultants with advanced-degrees. Theranostech's operations are located in a 19,000 sq. ft. facility in Albuquerque, which include 4,800 sq. ft. of newly-constructed state-of-the-art clean rooms.

Theranostech's management team has a broad range of experience in the field of business, biomedical research, quality control, regulatory affairs, production, R&D and sales and marketing. A seven-member Board of Directors has been formed and the Scientific Advisory Board is forming now.

Currently, Theranostech is focused on manufacturing and marketing of a proprietary, patented, cell-based technology platform called Tagger™. The company will be licensed to two US patents and two pending applications protecting the technology. This technology is targeted toward the diagnosis and monitoring of a variety of viral diseases and cancers. Because Tagger™ products are designed to monitor the progress of diseases, the results can be used to guide therapeutic treatment. Tagger™ represents one of the first technology platforms to incorporate both disease diagnosis and therapeutic monitoring features.

Theranostech's assay technology was conceived in 1996 as a test with applications in research and clinical assessment of HIV and other diseases. This was recognized in 1997 by the National Institute for Allergy and Infectious Diseases when it invited participation of our current lead product, gp120 Tagger™ - HIV, in a workshop for AIDS vaccine technology development.

The gp120 Tagger™ - HIV has already undergone four different pre-clinical trials where the product has been proven to detect and monitor HIV infection. As a cell-based, monoclonal antibody technology platform, Tagger™ has the ability to differentiate normal cells from diseased cells. This differentiation is accomplished by attaching "chemical light bulbs" to antibodies that bind specifically to diseased cells but not normal cells. The antibodies attach to specific parts of the diseased cells and label them. Then the cells are counted using standard laboratory equipment. Current test results support Theranostech's gp120 Tagger™ - HIV as being more informative and valuable than existing tests in ways that could provide major improvements in HIV drug development and usage. The success of these improvements is hoped to lower therapy costs, improve and extend lives.

The first gp120 Tagger™ - HIV kits have been manufactured, tested and final adjustments to the test are under way. Clinical trials for FDA approval are scheduled to begin during the in the first quarter of 2006. Theranostech intends to penetrate the monitoring market first.

Theranostech is seeking \$8,000,000 to start and complete the FDA approval process prior to June of 2007. A break even point will be reached within six months of FDA approval and projected sales are expected to reach \$5.8 million in year one, \$18.4 million in year two and \$34.2 million in year three, after FDA approval.

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FN: # gp120 + CD4 CRUS  
# CD4+ CRUS

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**In the Claims**

The following presentation of Claims replaces all previous versions.

We claim:

1. (currently amended) A method of assessing the infectivity status of a host infected with HIV, from a sample taken from the host, comprising:
  - a. measuring independently of each other the number of cells in the sample which are expressing cell-surface gp120 and the number of lymphocytes in said sample which are CD4 positive;
  - b. combining the result of the measurement of the number of cells expressing cell-surface gp120 and the result of the measurement of the number of lymphocytes which are CD4 positive;
 wherein the infectivity status of the host is assessed from the combination of the result of the measurement of the number of cells expressing cell-surface gp120 and the result of the measurement of the number of lymphocytes which are CD4 positive.
2. (original) A method of claim 1, wherein the infectivity status is represented by the number of cells expressing cell-surface gp120 per unit volume divided by the number of cells which are CD4 positive per unit volume.
3. (original) A method of claim 1, wherein the measuring is accomplished by flow cytometry.
4. (previously presented) A method of wherein the measuring is accomplished by a fluorescence resonance energy transfer assay. [FRET]
5. (original) A method of claim 1, wherein the cells are peripheral blood mononuclear cells.
6. (original) A method of claim 1, further comprising: combining an effective amount of an anti-gp120 antibody attached to a first detectable label and an effective amount of an anti-CD4 antibody attached to a second detectable label under conditions effective for said antibodies to bind gp120 and CD4 respectively.
7. (original) A method of claim 6, wherein said measuring is accomplished by flow cytometry.
8. (currently amended) A method of detecting lymphocytes expressing cell-surface gp120 in an aqueous sample containing viral infected cells displaying gp120, comprising:
  - a. combining to form a mixture:
    - i. an effective amount of a first antibody, comprising an anti-gp120 antibody, wherein the first antibody is attached to a detectable label,
    - ii. an effective amount of a second antibody, comprising an label-antibody specific for said detectable label, wherein said label-second antibody is attached to a magnetic particle, and
    - iii. the sample;
  - b. incubating said mixture under conditions effective for (i) binding of said anti-gp120 first antibody to gp120 on said cells, and (ii) for binding of said second antibody specific for said detectable label to said detectable label attached to said anti-gp120 antibody, to form a complex, wherein said anti-gp120 first antibody is bound to said gp120 displayed on a viral infected cell;
  - c. separating said complex by applying a magnetic field to said mixture, whereby said complex is retained by said magnetic field, and
  - d. determining the presence of magnetically separated lymphocytes expressing cell-surface gp120.
9. (original) A method of claim 1, wherein the CD4 count of said host is less than 200/mm<sup>3</sup> of whole blood.
10. (original) A method of claim 1, wherein the host has been treated with HAART.
11. (currently amended) A method of determining the infectivity status of a host infected with HIV virus who has tested negative in a virus co-culture assay, comprising: measuring the fraction of lymphocytes expressing cell-surface gp120 and the fraction of lymphocytes which are CD4 positive, and assessing the infectivity status of the host from a combination of the measurements of the two fractions.
12. (original) A method of claim 11, wherein the measuring is accomplished by flow cytometry.
13. (original) A method of claim 11, wherein the measuring is accomplished by a fluorescence resonance energy transfer assay.
14. (original) A method of claim 11, wherein the cells are peripheral blood mononuclear cells.

Response to 1/25/2005 Office Action; Application No. 09/893,604; page 2

anti-gp120 mAb  
FLOW CYTOMETRY

PAT 5817458 Mag Part.

FRET/FACS

HOW ARE MEASUREMENTS COMBINED?

DAUERL

I

DAUERL

102/103

ART

II

DAUERL

CLAIM REVISIONS

HOW? WHAT RATED?

gp120 PL  
CD4 PL

I

MAGNETIC  
PARAMAGNETIC

103  
DAUERL

HOW?

- ✓  
X15. (original) A method of claim 11, further comprising: combining an effective amount of an anti-gp120 antibody attached to a first detectable label and an effective amount of an anti-CD4 antibody attached to a second detectable label under conditions effective for said antibodies to bind gp120 and CD4 respectively. ✓  
X16. (original) A method of claim 15, wherein said measuring is accomplished by flow cytometry. ✓  
X17. (canceled)
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Daniel: Identified  $CD4^+/gp120^+$  cell population in biological samples; used dual FACS ( $\alpha$ -CD4 +  $\alpha$ -gp120)

p.4/same - gp120 measurements are standard

p.5/ " - CD4 " "

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COMMING ET AL (1999)

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**This application claims the benefit of U.S. Provisional Application Serial No.**

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(i) HIV-1-INDUCTED CD4 CELLS (I)  $\frac{(I)}{(T)} = \text{I.S.}$   
(ii) TOTAL CD4 CELL POPULATION (T)

infected cells and the number of cells which are capable of being infected by HIV, whereby the infectivity status of the host is assessed. These fractions can be presented as a ratio, e.g., the number of cells per unit volume which express cell-surface gp120 (productively-infected cells) divided by the number of cells per unit volume which are CD4 positive (cells capable of being HIV-infected).

The phrase "infectivity status" is intended as a description of the condition of a host with respect to the HIV virus, e.g., how many cells are actually infected with the HIV virus in comparison to the total number of cells which are capable of being infected. Such a value can be used to accurately describe the disease status of a patient, and determine the efficacy of treatment. Heretofore, CD4 and viral RNA markers (e.g., pol or gag) have been used as surrogates for assessing the disease status of a patient and the efficacy of treatment. However, CD4 positive cell counts have remained low in some patients, despite low levels of plasma RNA virus. See, e.g., Patterson et al., Lancet, 353:211-212, 1999. Thus, viral RNA is not always a good indicator of disease or treatment efficacy. A gp120/CD4 positive cell ratio in accordance with the present invention provides a superior value for assessing patient treatment and HIV disease progression. For example, a patient who had been shown to have low systemic CD4 positive cells and low RNA virus, may have measurable numbers of gp120 positive cells, indicating that HAART (highly active antiretroviral therapy) therapy should be continued. If this patient had been receiving, e.g., a protease inhibitor and two reverse transcriptase inhibitors, the detection of measurable numbers of gp120 cells indicates that the viral infection is continuing, and that the HAART therapy should be increased, e.g., by increasing dosages and/or by adding additional antiretroviral agents.

A "productively infected" cell means a cell which is infected by the HIV virus and which is actively producing functional virus. Any marker associated with the viral replication cycle can be used to measure productively infected cells, such as the presence of cell-associated viral RNA or antigens. Assays for each class of markers are well-known. For instance, viral RNA can be measured by branched DNA (bDNA) signal amplification [C. Pachl et al., "Rapid and precise quantification of HIV-1 RNA in plasma using a branched DNA (bDNA) signal amplification assay," *J. Acquir. Immune Def.*

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*Syndrom Hum. Retrovirol.*, 8:446-454 (1995); D. Kern et al., "An enhanced sensitivity branched DNA assay for quantification of human immunodeficiency virus type 1 RNA in plasma," *J. Clin. Microbiol.*, 34:3196-3202 (1996)], RT-PCR [K.B. Mullis et al., "Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction," *Methods Enzymol.*, 155:335-350 (1987); R.K. Saiki et al., "Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase," *Science*, 239:487-491 (1988)], QC-PCR [M.J. Piatak et al., "High levels of HIV-1 in plasma during all stages of infection determined by competitive PCR," *Science*, 259:1749-1754 (1993); M.J. Piatak et al., "Quantitative competitive polymerase chain reaction for accurate quantitation of HIV DNA and RNA species," *BioTechniques*, 14:70-79 (1993)], or nucleic acid sequence based amplification ("NASBA") [B. van Gemen et al., "Quantification of HIV-1 RNA in plasma using NASBA TM during HIV-1 primary infection," *J. Virol. Methods*, 43:177-188 (1993)]. See, also, e.g., *The AIDS Knowledge*, Chapter 2.4, "Viral Load Assays," published 1998 (<http://kali.ucsf.edu/akb/1997/02qrna/index.html>). Polypeptide antigens that can be utilized as markers for productive infection, include, e.g., gp120, gp41, and p24. p24 can be measured according to any effective assay, e.g., ELISA or Western blot. Assays for p24 can be performed as described in, e.g., *The AIDS Knowledge*, Chapter 2.3 "Tests to Detect HIV Antigen," published 1998 (<http://kali.ucsf.edu/akb/1997/02agtest/index.html>), [J. McKeating, "Quantitative assays for virus neutralization," In: Karn J., ed. *HIV: A practical approach*, *Virology and Immunology*, vol. 1. Oxford: IRL Press at Oxford University Press, pp. 118-127 (1995); P. Nishanian et al., "A simple method for improved assay demonstrates that p24 antigen is present as immune complexes in most sera from infected individuals," *J. Infect. Dis.*, 162:21-28 (1990); AABB Association Bulletin #96-2: HIV-1 antigen Test Implementation Guidance, January 5, 1996]. Productively infected cells can also be determined by quantitative coculture of peripheral blood mononuclear. This assay measures the number of HIV infected cells in a sample by determining the ability of sample cells to infect naive, uninfected cells. See, e.g., Hollinger et al, *J. Clin. Micro.*, 30:1787-1794, 1992.

In preferred embodiments of the present invention, the number of productively-infected cells in a sample is determined by the presence of cell-associated gp120. gp120 as a marker for productively infected cells has unexpected advantages over other assays and markers. For instance, gp 120 provides higher accuracy and ease of measurement than viral nucleic acid markers, such as those measured by PCR-based assays, quantitative coculture, and assays for other protein antigens, such as p24. In addition, gp120 is a more reliable measure of productive infection since it is a functional marker of viral replication. Nucleic acid-based assays, for instance, detect functional and non-functional, defective virus, giving misleading information about whether active infection is ongoing.

Cell-associated gp120 is can be measured by any effective assay. For instance, cells bearing cell-surface gp120 can be counted by labeling cells with an immunofluorescent marker, such as FITC, PE, or rhodamine, followed by counting the labeled cells, e.g., using a flow cytometer, on a hemocytometer, etc. Prior to counting, the cells can be separated, e.g., by magnetic separation techniques, e.g., as described in U.S. Pat. No. 5,817,458. An assay for cell-surface gp120 can comprises one or more of the following steps in any effective order: combining an effective amount of an anti-gp120 antibody attached to a detectable label, an effective amount of an antibody specific-for said detectable label, and an aqueous sample containing viral-infected cells displaying said gp120 to form a mixture, wherein said antibody specific-for said detectable label is attached to a magnetic particle; incubating said mixture under conditions effective for binding of said anti-gp120 antibody to gp120 on said cells, and, for binding of said antibody specific-for said detectable label to said detectable label attached to said anti-gp120 antibody, to form a complex, wherein said anti-gp120 antibody is bound to said gp120 displayed on a viral-infected cell; separating said complex by applying a magnetic field to said mixture, whereby said complex is retained by said magnetic field, and determining the presence of magnetically-separated cells by detecting said detectable label, whereby said magnetically separated cells are peripheral blood mononuclear cells expressing cell-surface gp120. Such an assay is described in U.S. Application Serial No. 09/139,663.

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The cells which are "capable of being infectedd by HIV" means, e.g., cells which express on their cell-surface the antigens which are utilized by the HIV virus to latch on to the cell in order to enter it. These antigens include, e.g., CD4, chemokine receptors, CCR5, CXCR4, CCR3, CCR2b, Bonzo, STRL33, BOB, GPR15, GPR1, US28, etc.

5 See, e.g., *Nature*, 388:230-231, 1997; *J. Virol.*, 71:1657-1661, 1997; Dean et al., *Science*, 273:1856-1862, 1996; E.A. Berger, 1997, *AIDS*, 11:S3-S16; Broder et al., 1997, *J. Leukocyte Biol.*, 62:20-29; Doms et al., 1997, *Virology*, 235:279-190; and Moore et al., 1997, *Curr. Opinion Immunol.*, 9:551-562. CD4 can be used alone, or in combination with any other antigen, such as a chemokine receptor, e.g., CCR5 or  
10 CXCR4.

CD4+ cells can be measured by any suitable means. For example, CD4 counts can be determined by immunotyping where detection of antigenic determinants specific to cell types is accomplished using labeled antibodies and generally a flow cytometer. These methods are widely utilized. See, e.g., 1997 Revised Guidelines for Performing CD4+ T-Cell Determinations in Persons Infected with Human Immunodeficiency Virus (HIV); Johnson et al., *J. Acquired Imm. Def.*, 10:522-530, 1995.

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Generally, immunotyping for the presence of CD4+ cells involves labeling cells with appropriate combinations of antibodies to distinguish CD4 T-cells from CD8 T-cells. CD4 T-cells can be identified as being positive for both CD3 and CD4; CD8 T-cells can  
20 be identified as being positive for both CD3 and CD8. CD45 can be further included to ensure that only lymphocytes are included. Two-, three- (Mecolino et al., *Cytometry*, 22:48-59, 1995; Nicholson et al., *Cytometry*, 26:227-230, 1996), or four-color assays can be utilized. CD3 can be used as common lineage marker for all lymphocytes, where other markers can be used to distinguish other classes, such as those mentioned above, and CD19  
25 for B-cells; CD16 for NK cells, granulocytes, and macrophages; CD56 for NK cells.

If a sample is whole blood, it may be desirable to treat or fractionate the sample prior to determining the number of CD4 positive and/or productively-infected cells. Whole blood comprises, e.g., serum, proteins, erythrocytes, leucocytes, platelets, etc. The leucocyte fraction includes, polymorphonuclear granulocytes, monocytes, and

lymphocytes. The erythrocytes can be lysed by any conventional method, such as using ammonium chloride.

The samples can be stained and fixed by conventional methods, and then immunotyped. If desired, the peripheral blood mononuclear cells ("PBMC", e.g.,  
5 monocytes and lymphocytes) are separated from the whole blood using a centrifugation procedure with an appropriate medium, such as Lymphocyte Separation Medium (ICN - Costa, Mesa, CA).

As mentioned, a preferred ratio in accordance with the present invention comprises the number of gp120 positive cells per unit volume/the number of CD4  
10 positive cells per unit volume. The determination of gp120-bearing cells can be made on the same or different sample as the one use to determine CD4+ cells. The number of CD4-bearing cells can be determined by the immunotyping procedures as described above.

During HIV infection, CD4 bearing cells can be under-estimated because the CD4  
15 antigen can be down-regulated in infected, or cells which have acquired gp120 from the plasma. Thus, in certain aspects of the invention, the CD4 positive count can be determined by measuring CD4 positive cells and then adding to it cells which are expressing gp120 antigen on their cell-surface. Such count can be referred herein as the "adjusted CD4 positive cell count."

20 Pools of receptive cells, and subsets of these pools which are infected with virus, can be determined in various ways. As described above, CD4 and gp120 expression are determined independently of each other. For example, the antigens can be labeled with different markers (e.g., using a FITC-conjugated antibody for a first antigen, and a rhodamine-conjugated antibody for a second antigen), and then examined for the presence  
25 of each marker.

Fluorescence resonance energy transfer ("FRET") systems can also be used to determine co-expression of a viral antigen and a cellular antigen present on viral receptive cells. In a FRET assay, an excited donor fluorophore transfers its energy to an acceptor fluorophore when the two are in proximity. This transfer is not an emission and absorption  
30 of light, but a non-radiative, direct energy transfer. The emission of a detectable signal

from the acceptor molecule indicates it is close to the donor. By labeling antibody types with different acceptor and donor fluorophores, it can be determined when the antibodies are recognized antigens which are co-expressed in the same cell. For instance, if anti-HIV antigen (e.g., anti-gp120) is labeled with a donor, and a cell-based antigen (e.g., CD4, CD45, etc.) is labeled with an acceptor, a FRET assay can be used to determine when both antigens are present on the same cell, or when only one antigen is present. In the latter case, the donor and acceptor molecules would have detectable signals which are different from the signal produced when the donor is in close proximity to the acceptor. There are many commercially available systems.

10 A suitable system for the present invention uses Renilla luciferase (Rluc) as the donor and a modified Green Fluorescent Protein (GFP2) as the acceptor molecule in an assay analogous to fluorescence resonance energy transfer (FRET), but without the need for an excitation light source. Rluc emits blue light between 390-400 nm upon addition of the substrate, DeepBlueC™. GFP2 absorbs this light and emits fluorescence at 505-510 nm that can be detected using a fluorimeter. If Rluc and GFP2 are in close proximity, due to binding of the biological partners, energy is efficiently transferred and both the blue light of Rluc and green light of GFP2 are detected. See, e.g., Xu et al., *Proc. Natl. Acad. Sci.*, 96:151-6, 1999; Angers et al., *Proc. Natl. Acad. Sci.*, 97:3684-9, 2000. Another commercially available technology that can be used is AlphaScreen™, an "Amplified Luminescent Proximity Homogeneous Assay" method. Upon illumination with laser light at 680 nm, a photosensitizer in the donor bead converts ambient oxygen to singlet-state oxygen. The excited singlet-state oxygen molecules diffuse approximately 250 nm (one bead diameter) before rapidly decaying. If the acceptor bead is in close proximity of the donor bead, by virtue of a biological interaction, the singlet-state oxygen molecules reacts with  
25 chemiluminescent groups in the acceptor beads, which immediately transfer energy to fluorescent acceptors in the same bead. These fluorescent acceptors shift the emission wavelength to 520-620 nm. The whole reaction has a 0.3 second half-life of decay, so measurement can take place in time-resolved mode. For other fluorophores, e.g., Carraway et al., *J. Biol. Chem.* 264(15):8699-707, 1989; green fluorescent protein and acceptor  
30 fluorophore Cy3. Donor and acceptor fluorophores can be routinely coupled to the appropriate antibody.

## EXAMPLES

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### **METHODS**

Studies made to establish sensitivity, specificity, positive predictive value, and accuracy (as compared with HIV culture) were performed in 62 patients who were known to be seropositive for HIV-1 and were in various stages of the HIV disease process.

10 Almost all were homosexual men between ages 25 and 65. Most of the subjects were taking antiretroviral therapy, some were about to commence therapy.

Clinical application was explored in a group of 45 patients within one clinical trial that compared the outcomes of HAART that comprised 3 drugs with those from HAART that comprised 4 drugs.

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15 The false positive rate was studied in 70 putatively HIV-negative individuals; these were recruited from among patients attending the Department of Medicine and from among members of the clinic staff. These were all presumed by the investigators to be HIV-negative and did not announce themselves to be HIV-positive when invited to participate as HIV-negative controls; we did not perform HIV antibody or antigen tests in  
20 these persons blood samples.

Three different monoclonal antibodies (Mabs) against HIV-1 gp120 were commercially obtained and were conjugated with fluorescein isothiocyanate. These three Mabs, used in combination, were identified as the optima amongst many that were tried both singly and in various combinations. The three were used in combination for labeling  
25 the lymphocytes.

Labeling of cells for flow cytometric analysis was conducted according to CDC guidelines for immunophenotyping assays. Whole blood was collected in EDTA, maintained at 18-22°C during transport, and testing was performed within 30 hours of the blood draw.



Flow cytometric analysis was made on a Becton Dickinson FACSCalibur flow cytometer using CELLQuest acquisition and statistical analysis software. Results were expressed as % of CD4+ cells expressing gp120 (gp120+/CD4+). ROC curves established 310% gp120+/CD4+ as the cut-off for defining a positive test.

5 Quantitative PBMC micro co-culture was made by the ACTG consensus protocol which uses limiting dilution culture data with a maximum likelihood method (DAIDS Virology Manual for HIV Laboratories, version 1/1/1997). Results are expressed as infectious units per million cells (IUPM).

10 The studies were approved by the Institutional Review Board and all subjects, both HIV-positive individuals and putatively HIV-negative persons, gave signed, informed consent.

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15 Without further elaboration, it is believed that one skilled in the art can, using the preceding description, utilize the present invention to its fullest extent. The preceding preferred specific embodiments are, therefore, to be construed as merely illustrative, and not limitative of the remainder of the disclosure in any way whatsoever.

The entire disclosure of all applications, patents and publications, cited above, including U.S. Application Serial No. 09/139,663 and PCT/US97/19849, are hereby incorporated by reference. U.S. Provisional Application Serial No. 60/215,075, filed June 20 30, 2001, is hereby incorporated by reference in its entirety

25 From the foregoing description, one skilled in the art can easily ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions.

## Serotyping of Primary Human Immunodeficiency Virus Type 1 Isolates from Diverse Geographic Locations by Flow Cytometry

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MIMI KIM,<sup>2</sup> JOHN MASCOLA,<sup>3</sup> AND FRANCINE MCCUTCHAN<sup>4</sup>

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The immunologic relatedness of the various human immunodeficiency virus type 1 (HIV-1) clades was determined with 13 human anti-HIV-1 monoclonal antibodies (MAbs) to six immunogenic regions of the HIV-1 structural proteins. The immunoreactivity of the native, oligomeric viral envelope glycoproteins expressed on the surfaces of human peripheral blood mononuclear cells infected *in vitro* with primary isolates from clades A through E was determined by flow cytometry. Some epitopes in the immunodominant region of gp41 and the C terminus of gp120 appear to be HIV-1 group specific in that they are expressed on the surfaces of cells in cultures infected with the majority of viruses tested from clades A to E. Epitopes within the V3 region appear to be clade restricted. Surprisingly, one MAb to an epitope in the C terminus of gp120 was entirely clade B specific. Staining with anti-V2 and anti-CD4 binding domain (CD4bd) reagents was infrequently detected. Anti-CD4bd MAbs stained only CD4-negative T cells because the CD4bd of gp120 appeared to be complexed with membrane CD4. When present, the epitopes of V2 and the CD4bd appeared to be expressed on cells infected with various clades. Thus, the results suggest that MAbs to gp41, the C terminus, and the V3 loop of gp120 are most useful in serotyping primary isolates of HIV-1, providing group-specific, clade-restricted, and clade-specific reagents. The use of the immunofluorescent method with the reagents described herein distinguishes infection with clade B from that with all other HIV-1 clades. With additional MAbs, this technique will allow a broadly applicable, reproducible, and practical method for serotyping HIV-1.

In order to map the immunologic relationships among the human immunodeficiency virus type 1 (HIV-1) clades and determine how serotypes parallel or diverge from genotypes, immunologic studies with primary isolates from the various HIV-1 clades and with defined monoclonal antibodies (MAbs) are required. These studies are also needed to characterize the virus within an individual and the virus strains within a defined population, and they are necessary, as well, to provide a foundation for ongoing vaccine development and information about the extent to which critical epitopes are shared by viruses of the various clades of HIV-1.

Preliminary studies of the immunologic relatedness of the various HIV-1 subtypes have been published. Thus, some group-specific epitopes in the HIV-1 envelope have been identified. These include determinants in the immunodominant region of gp41 (6, 53) and the C terminus of gp120 (43). While MAbs to these epitopes have not previously been tested on typed viruses from around the world, these epitopes appear to be present in all or most variants of HIV-1 that have been tested to date.

Antibodies to the CD4 binding domain (CD4bd) of gp120 are also commonly referred to as being group specific since early studies indicated that antibodies to this region cross-react with divergent laboratory isolates (22). Antibodies to the CD4bd have neutralizing activity, but anti-CD4bd MAbs are not capable of neutralizing all HIV-1 strains; thus, of a panel of three human anti-CD4bd MAbs tested against eight type B HIV-1 laboratory isolates, none was able to neutralize all vari-

ants (16). Similar results were found by others using both laboratory and primary isolates of HIV-1 (2, 24, 39, 44, 51).

A potentially important group-specific MAb is that reported by Muster et al. (37). This human MAb is directed against the external domain of gp41 in a region that is highly conserved; it is capable of neutralizing many type B isolates of HIV-1 (10) but has not been tested for its ability to react with non-clade B viruses.

To date, no clade-specific antibodies reactive with envelope antigens have been definitively identified. The data of Mascola et al. (32) suggest that polyclonal sera from infected individuals show clade-restricted neutralization patterns in that sera from U.S. and Thai patients preferentially neutralize the homologous clades of U.S. and Thai primary isolates. One human MAb and some rodent anti-V3 (clade B) antibodies (1, 9, 16, 41) appear to react broadly with many clade B viruses, but there are, as yet, no reports as to their reactivity with non-clade B viruses.

Intracode subdivisions have been distinguished genotypically. Thus, for example, clade F viruses have been divided into two separate categories, with one subclade appearing in Brazil and one occurring in Romania (11, 31, 45). Similarly, intracode groups have been identified serologically. Thus, Ebenbichler et al. (12) have used a panel of anti-V3 MAbs to classify two distinct V3 loop conformations which correlate with T-cell tropism and macrophage tropism.

Most of the aforementioned studies of immunologic relationships among HIV-1 viruses have been performed with laboratory isolates or with untyped primary isolates. In order to define these relationships more precisely, we have chosen to use human MAbs to analyze the expression of epitopes on peripheral blood mononuclear cells (PBMCs) infected *in vitro* with primary viruses from five different clades. These human

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CD4/gp120 DIRECTION / FACS (NOT FROM PATIENT THOUGH)  
- NOT DATA!

MAbs were induced by native viral proteins during the course of natural infection and were selected on the basis of their reactivity with proteins or peptides of laboratory strains of clade B. Using these methods and materials, we have been able to define group-specific, clade-restricted, and clade-specific epitopes of HIV-1.

## MATERIALS AND METHODS

**Viruses used.** Virus preparations were provided by three sources: (i) the Military Medical Consortium for Applied Retroviral Research and the Henry M. Jackson Foundation for Military Medicine (MMCARR/HMJF), (ii) the World Health Organization (WHO), and (iii) the National Institute of Allergy and Infectious Diseases (NIAID). The methods of virus isolation and preparation have been described previously (7, 47). Briefly, virus was isolated by coculture of phytohemagglutinin (PHA)-stimulated PBMCs from infected and uninfected individuals. For expansion to high-titered stocks, culture supernatants from the initial isolates were used to infect PHA-stimulated PBMCs from healthy, uninfected individuals. Cultures were monitored for p24 production, and supernatant was collected and stored in 1-ml aliquots in liquid nitrogen. None of the primary isolates was passaged through neoplastic cell lines, and the viruses used throughout were uncloned; each virus preparation therefore contains a population of viruses. A PBMC-adapted stock of HIV<sub>LA1</sub> was obtained from A. Prince. This virus was prepared and tested in the same manner as that described above for primary isolates.

The assignment of each virus to a clade was based on multiple genetic analyses (54). Amino acid sequences were described by Myers et al. (38) or were provided by MMCARR/HMJF, WHO, or NIAID. Viruses received from WHO and NIAID are referenced according to the nomenclature now used by Myers et al. (38). Thus, for example, isolate A2RW021 denotes that this isolate belongs to clade A (A), was collected in 1992 (indicated as 2), was derived from a patient in Rwanda (RW), and was assigned specimen number 021. Isolates provided by MMCARR/HMJF do not conform to this identification scheme. Country codes include RW, Rwanda; US, United States; BZ or BR, Brazil; TH, Thailand; HA, Haiti; SM, Somalia; SG, Senegal; and UG, Uganda. The designations for virus phenotypes were provided by each of the contributing agencies. In cases in which analyses of syncytium-inducing and non-syncytium-inducing phenotypes had not been performed by the virus provider, such analyses were performed as part of this study by means of the MT-2 assay of Koot et al. (30).

The following virus isolates were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, from the WHO Network for HIV Isolation and Characterization: A2RW021W, A2RW023W, A2RW026W, B2TH014W, B2TH026W, C2BR025W, D2UG001W, D2UG021W, D2UG024W, E2TH001W, E2TH003W, and E2TH005W.

The following isolates were obtained through the Division of AIDS, NIAID: B2US657D and B2US727D from the Multicenter AIDS Cohort Study, from Roger Detels and John Phair, respectively; B2HA593D, B2HA594D, and B2HA599D from Neal Halsey, Johns Hopkins University; and B2US714D from Kenrad Nelson, Johns Hopkins University.

**Preparation of cultured, infected cells for flow cytometry.** PBMCs from uninfected donors were prepared by Ficoll-Hypaque density centrifugation from Leukopaks purchased from the New York Blood Center. The Leukopaks consisted of whole blood, collected in CPDA-1 (citrate, phosphate, dextrose, and adenine solution), from which plasma had been removed. These fresh PBMCs, at a concentration of  $2 \times 10^6$  cells per ml, were stimulated for 3 days with 5  $\mu$ g of PHA per ml (Difco Laboratories, Detroit, Mich.) in medium containing 20% fetal calf serum, 2% penicillin-streptomycin (Bio-Whittaker, Walkersville, Md.), and 1% L-glutamine (Bio-Whittaker). Cultures were carried out in tissue culture flasks incubated in 7% CO<sub>2</sub> at 37°C. After 3 days, the cells were readjusted to a concentration of  $2 \times 10^6$  cells per ml in the medium described above, except that the medium lacked PHA and was supplemented with 20 U of recombinant interleukin-2 per ml (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Cells (5 ml) and virus (1 ml) containing a minimum of 20 50% tissue culture infectious doses per ml were incubated together for 30 min at 37°C, and then 4 ml of interleukin-2-supplemented medium was added. The cultures were maintained with feeding every 2 days, spun, decanted, and resuspended in 10 ml of medium (as delineated above) until the number of CD4-positive cells decreased by at least 10% as measured by flow cytometry (see below), usually 4 to 8 days. The same procedure was used for primary isolates and for PBMC-adapted HIV<sub>LA1</sub>. For uninfected negative control cultures, virus-free medium was added in place of virus; all other steps in infected and uninfected cultures were identical.

**Antibodies used for staining.** The mouse anti-CD4 MAb Leu-3a (Becton Dickinson, San Jose, Calif.) was used to identify CD4-positive cells. The human anti-HIV-1 MAbs used for analysis and the epitopes for which they are specific are shown in Table 1. Human MAbs were used to determine if antibodies representative of those produced by the human immune response could distinguish between viruses from different geographic locations and if they could define serotypes. The human MAbs used were chosen from a panel of over 60. They were selected on the basis of the diversity of epitopes with which they react,

TABLE 1. Human MAbs used

MAb	Epitope	Reference(s)
71-31	p24	17
50-69	gp41 (amino acids 579-613)	17, 56
858-D	gp120 (C terminus: VVQREKR)	This study
670-D	gp120 (C terminus: PTKAKRR)	This study
989-D	gp120 (C terminus: VVQREKR)	This study
257-D	gp120 (V3 loop: RKIH)	19, 20
268-D	gp120 (V3 loop: HIGPGR)	19, 20
447-52-D	gp120 (V3 loop: GPGR)	16, 20
694/98-D	gp120 (V3 loop: GRAF)	16, 20
697-D	gp120 (V2 loop)	18
559/64-D	gp120 (CD4 binding domain)	28
654-D	gp120 (CD4 binding domain)	33
729-D	gp120 (CD4 binding domain)	33

and those used were MAbs whose epitopes had been well characterized (Table 1 and references therein). Maximal staining was achieved at MAb concentrations of  $\geq 0.5$   $\mu$ g/ml. Staining levels remained constant when MAb concentrations of up to 80  $\mu$ g/ml were used. Three human MAbs to the C terminus of gp120 which have not previously been described were used in these studies. Each of these was produced by heterohybridomas derived from the PBMCs of HIV-infected individuals as previously described (17, 19), and they were selected by screening supernatants on recombinant gp120 (rgp120) from HIV<sub>LA1</sub> (Repligen, Cambridge, Mass.). The anti-C terminus MAbs were mapped by their ability to react with peptides from the C terminus of rgp120 (data not shown).

Unpurified MAbs in culture supernatants were used to avoid any aggregation which might occur upon purification and which might lead to an increase in nonspecific background staining levels. A pool of sera from five HIV-positive subjects was also used to stain cells; this serum pool was used at a dilution of 1:1,000.

**Staining of cells and flow cytometric analysis.** For single-color staining,  $10^6$  washed cells (in 100  $\mu$ l) which had been cultured as described above were incubated with 100  $\mu$ l of a given human MAb for 30 min at 4°C. Following incubation, the cells were washed once by centrifugation in phosphate-buffered saline containing 0.1% sodium azide (PBS-azide). To the cell pellet was added 50  $\mu$ l of a 1:8 dilution of phycoerythrin (PE)-conjugated goat Fc-specific anti-human immunoglobulin G (IgG) (Caltag, South San Francisco, Calif.). After 30 min of incubation at 4°C, the cells were again washed once, resuspended in PBS-azide, and analyzed by flow cytometry with a FACScan (Becton Dickinson). For single-color analyses, the cells were gated on live lymphocytes using forward and 90° scatter; the negative peak was defined with infected cells stained with goat anti-human IgG-PE in the absence of any human anti-HIV MAb.

For two-color analyses, the cultured cells were incubated with 100  $\mu$ l of the designated human anti-HIV MAb and 15  $\mu$ l of fluorescein isothiocyanate-labeled mouse anti-CD4 MAb (Becton Dickinson) for 30 min at 4°C and then were washed once with PBS-azide. A 1:8 dilution of the goat anti-human IgG-PE (50  $\mu$ l) was then added, incubation was continued for 30 min at 4°C, and after they were washed once, the cells were resuspended and analyzed. For dual-color analyses, the cells were gated on scatter as described above. The cells were gated on CD4-positive cells to determine the mean channel fluorescence of CD4-positive cells stained with anti-HIV MAbs.

For three-color analyses, in addition to human anti-HIV MAbs and fluorescein isothiocyanate-labeled anti-CD4, Per CP anti-CD3 (Becton Dickinson) was added to the cultured cells in the initial incubation. The cells were gated by scatter and regated on HIV-positive, CD4-negative cells to determine the percentage of the latter which were CD3 positive.

Flow cytometry was carried out with a Becton Dickinson FACScan. The instrument sensitivity and reproducibility were verified with reference particles (Calibrite Beads; Becton Dickinson). Compensation was set with fluorochrome-labeled reference particles and optimized on normal control cell mixtures. Data on one to three experiments performed with each virus are reported. When more than one experiment was performed (for 8 of the 28 viruses studied), data are shown as the mean; repeat analyses were performed on newly infected cells. Each analysis was performed on cells infected with viruses as they were received, and thus viruses of several different clades were usually represented in each experiment that was conducted.

**Statistical methods.** The MAbs were grouped into clusters by the techniques of cluster analysis (26). Similarities between MAbs were based on the patterns of response (positive and negative) of cells infected with HIV isolates to the MAbs, and these similarities were quantified by the average linkage algorithm. This algorithm treats the distance between two clusters as the average distance between all pairs of items for which one member of a pair belongs to each cluster. Initially, all MAbs are in separate clusters. The most similar MAbs are then grouped, and these initial groups are subsequently merged according to their



FIG. 1. Percentages of cells in cultures infected with primary isolates (or HIV<sub>LAI</sub>) which stain positively with human MABs. Values in color are in excess of the mean value + 2 standard deviations for cells in infected cultures stained with anti-p24 as a negative control (3.39% + 2.8% = 6.19%). C-TERM, C terminus; NSI, non-syncytium inducing; SI, syncytium inducing; N.D., not done.

similarities. Eventually, all subgroups are fused into a single cluster. SAS statistical software was used for the analysis.

## RESULTS

**Staining characteristics of HIV<sub>LAI</sub>-infected cells.** To ascertain the ability of a panel of MABs to stain antigens on the surfaces of HIV-1-infected cells, PBMCs infected with a PBMC-adapted variant of HIV<sub>LAI</sub> were stained with each of 13 human MABs. An anti-p24 MAB (MAB 71-31) was used as a negative control. With this latter MAB, only 2.9% of cultured cells were stained over the gate set with infected cells which were treated with goat anti-human IgG-PE in the absence of any human anti-HIV MAB. Anti-V3 MABs 257-D and 268-D had previously been shown to be unreactive with HIV<sub>LAI</sub> (19, 20); these two MABs served as additional negative controls in this experiment and were found to stain 2.6 and 2.5% of infected cells, respectively. In 15 experiments, staining levels with the anti-p24 MAB used as a negative control reached a mean + 2 standard deviations of 6.2%, which was hereinafter used as the cutoff between negative and positive values. The additional 12 anti-HIV MABs tested, which were specific for antigenic determinants of gp41 and gp120, had previously been shown to react with HIV<sub>LAI</sub> (16, 17, 19, 20, 28), and each stained >6.2% of cells (Fig. 1). Three anti-CD4bd MABs known to react with rgp120 HIV<sub>LAI</sub> stained between 7.0 and 8.0% of cells. An anti-V2 MAB (697-D) stained 9.2% of cells, and an anti-gp41 MAB (50-69) stained 27.1% of cells. Two MABs to the V3 loop, 447-52-D and 694/98-D, known to react with and neutralize HIV<sub>LAI</sub>, stained 19.1 and 58.4% of cells in HIV<sub>LAI</sub>-infected cultures, respectively. The differential staining of these two anti-V3 MABs is consistent with their respective binding affin-

ities for rgp120<sub>LAI</sub> and their ability to neutralize HIV<sub>LAI</sub> (19, 20). Other MABs which were also reactive with the cells in the HIV<sub>LAI</sub>-infected culture were the MABs to the C terminus of gp120 (MABs 670-D, 858-D, and 989-D), which stained 62.8, 57.6, and 48.3% of the cells, respectively. Thus, among the reactive MABs, the hierarchy of surface staining by antibody specificity for various epitopes was (in order of ascending percentages of cells stained) anti-CD4bd < anti-V2 < anti-gp41 < anti-V3 ~ anti-C terminus. Uninfected cells did not stain above background levels with any anti-HIV MAB (Fig. 1) but did stain with anti-CD4 (data not shown).

The hierarchy of staining ability was similar to that derived when the data were analyzed for mean channel fluorescence (MCF) rather than for percent positive cells. Thus, the average MCF value for the three MABs that did not stain the cells was 37.3. The MCF value for three anti-CD4bd MABs was 39, that for one anti-V2 MAB was 49, that for one anti-gp41 MAB was 70, that for two anti-V3 MABs was 86, and that for three anti-C terminus MABs was 116.

Because the anti-CD4bd MABs showed only a small percentage of positively stained cells (7.0 to 8.0%) and the MCF value of CD4-positive cells stained with anti-CD4bd MABs was essentially unchanged above the negative control values, the nature of the cells binding the anti-CD4bd and other anti-HIV MABs was investigated. Figure 2a shows that neither CD4-negative nor CD4-positive cells stained with the anti-p24 MAB 71-31. Figure 2b and c show that both CD4-negative and CD4-positive cells stained intensely with the anti-V3 and anti-C terminus MABs, respectively. However, Fig. 2d, e, and f show that three anti-CD4bd MABs stained only CD4-negative cells. These data suggest that on CD4-positive cells, the CD4bd of

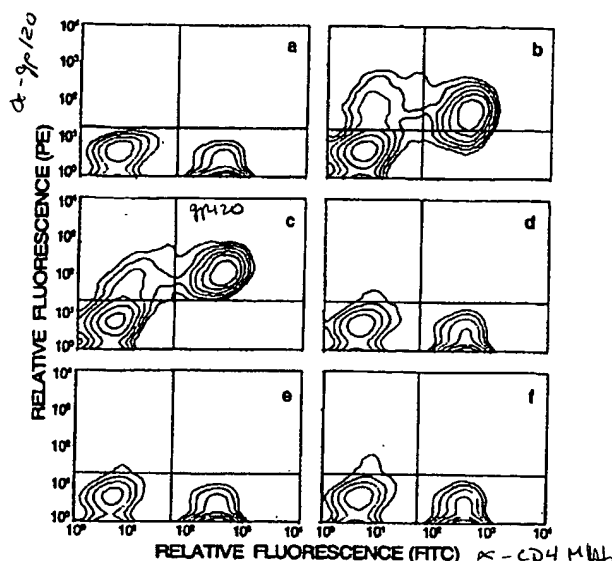


FIG. 2. Relationship of CD4 and HIV antigen expression determined by flow cytometric analysis of PHA-stimulated PBMCs which had been infected 4 days earlier with a PBMC-adapted strain of HIV<sub>LAI</sub>. Contour maps of cells stained with fluorescein isothiocyanate (FITC)-labeled anti-CD4 mouse MAb and with human anti-HIV MABs and PE-conjugated goat Fc-specific anti-human IgG are shown. The intensity of the fluorescein isothiocyanate staining (anti-CD4) is shown on the x axis, and the intensity of the PE staining (anti-HIV) is shown on the y axis. Human anti-p24 MAb 71-31 (a), anti-V3 MAb 694/98-1 (b), anti-C terminus (gp120) MAb 670-D (c), anti-CD4bd MAb 559/64-D (d), anti-CD4bd MAb 654-D (e), and anti-CD4bd MAb 729-D (f) were used.

gp120 may be complexed with CD4, thus blocking the CD4bd. However, since HIV-1 infection down-regulates CD4 (15, 25, 48), the CD4bd should be available on infected cells which no longer express CD4. This appears to be supported by the data shown.

**Staining characteristics of cultures infected with primary isolates.** In order to ascertain whether cells in cultures infected with primary isolates were stained in a pattern similar to that observed with the cells infected with the HIV<sub>LAI</sub> clade B laboratory strain, normal PBMCs were infected with one of several clade B viruses. The results, summarized in Fig. 1, show that cells in cultures infected with primary isolates of clade B can, indeed, be stained with anti-gp41, anti-C terminus, anti-V3, anti-CD4bd, and anti-V2 MABs. The details of the staining profiles with these reagents are presented in Fig. 1 and are described below. Figure 3 shows the contour maps of cells in cultures infected with a clade B virus, B2HA593D, which were stained with anti-CD4 and various anti-HIV MABs. The similarity of the staining patterns of cells in cultures infected with this primary isolate to those of cells infected with the PBMC-adapted strain of HIV<sub>LAI</sub> is evident from a comparison of Fig. 2 and 3. Thus, essentially no cells were stained with anti-p24 (Fig. 2a and 3a), cells were stained strongly with anti-V3 and anti-C terminus MABs among both CD4-negative and CD4-positive populations (Fig. 2b and c and 3b and c), and CD4-negative cells were essentially the only cells which were stained with the anti-CD4bd MABs (Fig. 2d to f and 3d to f). Three-color analysis demonstrated that 88% of the infected CD4-negative cells were CD3-positive T cells (data not shown). Moreover, time course experiments showed that the CD4bd on CD4-negative cells was the last epitope to be detected, and this occurred at a time when the number of cells expressing CD4

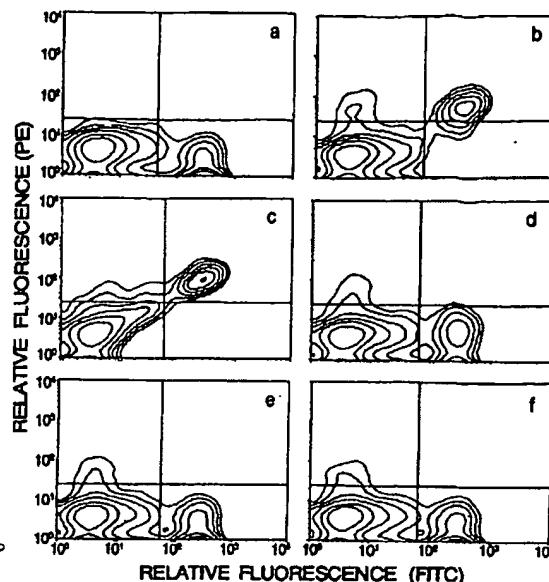


FIG. 3. Relationship of CD4 and HIV antigen expression determined by flow cytometric analysis of PHA-stimulated PBMCs which had been infected 4 days earlier with primary isolate B2HA593D. Contour maps are shown, and the details of the staining are identical to those delineated in the legend to Fig. 2. FITC, fluorescein isothiocyanate.

had begun to decline dramatically (data not shown). These data support the hypothesis that the CD4bd is blocked by membrane CD4 but is available for binding on cells whose CD4 has been down-regulated.

The staining intensity of the primary isolates with the different categories of MABs was analyzed next. For those CD4-positive cells from infected cultures that were stained positively with anti-HIV MABs, staining was weakest with anti-CD4bd MABs, intermediate with anti-gp41 and anti-V2 MABs, and strongest with anti-C terminus and anti-V3 MABs. Examples of the intensity of the staining of the cells infected with clade B primary isolates can be discerned from the contour maps in Fig. 3 and from the histograms in Fig. 4. It should be noted that the intensities of staining achieved with polyclonal antibodies from a pool of sera from HIV-infected individuals (Fig. 4g), with an anti-C terminus MAB (Fig. 4e), and with an anti-V3 MAB (Fig. 4f) were similar (MCFs were 138.1, 128, and 133.5, respectively).

**Group-specific antigens recognized on the surfaces of cells in cultures infected with HIV-1 primary isolates from diverse locales.** After the ability of various human anti-HIV MABs to stain PBMCs in cultures infected with laboratory and primary isolates of clade B was established, the ability of these MABs to stain PBMCs in cultures infected with various primary isolates from diverse geographic locations was tested. Virus isolates from clades A to E were used to infect PHA-stimulated human PBMCs. Three MABs were found to stain cells in cultures infected with most of the viruses tested (Fig. 1). Thus, MAb 50-69 specific for the immunodominant domain of gp41 (56) stained cells in cultures infected with 21 of 27 (78%) of the primary viruses tested. Similarly, MABs 670-D and 858-D specific for the C terminus of gp120 stained cells in cultures infected with 100% ( $n = 19$ ) and 81% ( $n = 27$ ) of the viruses tested, respectively. In each case, cells in cultures infected with viruses from each of the five clades were among those which

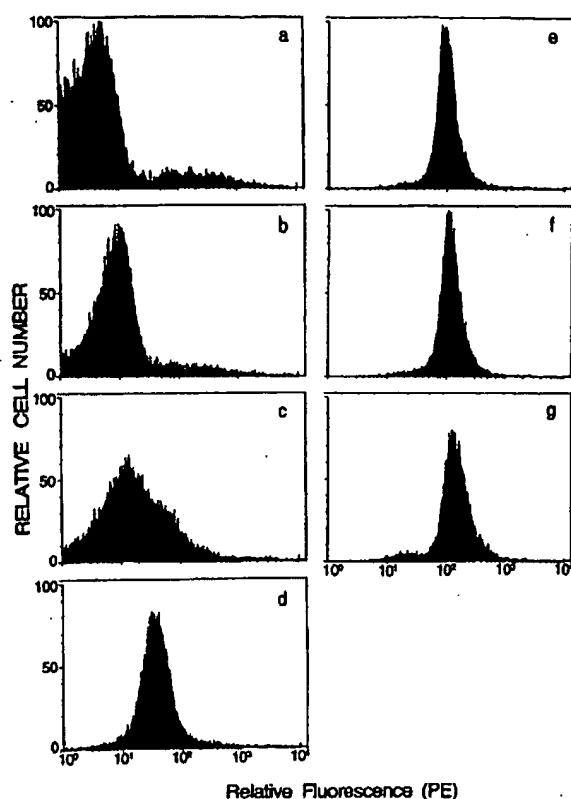


FIG. 4. Fluorescence histograms of CD4-positive PHA-stimulated PBMCs which had been infected 4 days earlier with primary isolate US1. The intensities of the staining with human anti-p24 MAb 71-31 (a), anti-CD4bd MAb 559/64-D (b), anti-gp41 MAb 50-69 (c), anti-V2 MAb 697-D (d), anti-C terminus (gp120) MAb 670-D (e), and anti-V3 MAb 447-52D (f) are shown. In panel g, the cells were stained with a 1:1,000 dilution of sera pooled from five HIV-positive subjects.

stained positively. These data confirm the findings of others that antigens from the immunodominant domain of gp41 and from the C terminus of gp120 appear to be ubiquitous among HIV-1 isolates and are, therefore, group-specific antigens (43, 53). The frequency with which cells from infected cultures stained positively with anti-C terminus MAbs ranged from 8 to 82% (Fig. 1). This is not dissimilar to previous reports that 50 to 100% of cells infected with HIV *in vitro* express antigens that can be detected by flow cytometry (5, 23, 29, 55).

**Clade-restricted antigens on the surfaces of cells in cultures infected with HIV-1 primary isolates.** Four anti-V3 MAbs were tested for their ability to recognize antigens on the surfaces of cells in infected cultures. These MAbs had been derived from the cells of North American HIV-positive patients and had been shown to react with the V3 loop from clade B viruses. These MAbs reacted primarily with cells in cultures infected with viruses from clade B (Fig. 1). Eleven of the 14 primary clade B viruses tested contain the GPGR motif at the crown of the loop. Staining this group of 11 primary isolates with the four anti-V3 MAbs resulted in positive values in 38 of 44 (86%) analyses. Of the three clade B viruses which had divergent sequences at the crown of the V3 loop, two were from Thailand (B2TH026W and TH237) and one was from Haiti (B2HA599D). The two Thai viruses differed from the majority

of viruses tested here by having PLGPGQ and HLGPGR (instead of HIGPGR) at the tip of the loop. The aberrant Haitian virus had a 3-amino-acid insertion near the crown of the loop and GGGR at the tip (Table 2). These viruses are hereinafter categorized as clade B' on the basis of their aberrant binding to the four anti-V3 MAbs. Only 1 of 12 (8%) analyses of these clade B' viruses resulted in cells stained positively with anti-V3 MAbs. The data suggest that most of the type B viruses from around the world, including those from the United States, Brazil, Haiti, and Thailand, can be identified with anti-V3 (clade B) human MAbs.

The anti-V3 MAb 447-52D, which is specific for the GPGR sequence at the crown of the V3 loop, reacted with cells infected with all of the viruses bearing this sequence (Table 2); this MAb has previously been shown to be broadly neutralizing for clade B primary and laboratory isolates (9, 16). MAb 694/98-D has GRAX as its core epitope; it stained cells infected with 10 of 11 viruses having this sequence in the V3 loop. This MAb, more than any of the other anti-V3 MAbs, is affected by conformation-dependent factors, as it was selected on rgp120 rather than on a V3 peptide (16, 20). MAb 257-D, whose core epitope was previously defined as KRIHI (19), stained cells in cultures infected with 9 of 11 clade B primary viruses; those not stained or which stained poorly (viruses BZ167 and B2US714D) were infected with viruses with a substitution in at least one residue within this epitope (Table 2). MAb 268-D, whose core epitope was previously defined as HIGPGR (19), stained cells in cultures infected with 8 of 11 clade B primary viruses; cells that were not stained were infected with viruses with a substitution for the histidine in this epitope (Table 2). These data show the remarkably consistent sequence-dependent staining by anti-V3 MAbs.

Two of the anti-V3 MAbs showed reactivity with cells infected with one of three clade A viruses. The staining of the cells cultured with this clade A virus (A2RW023) was strong and unequivocal in three replicate experiments.

One anti-C terminus MAb, 989-D, was entirely clade B specific but was reactive with only 7 of 11 (64%) clade B primary viruses and with two of three (67%) clade B' viruses.

**Expression of V2 on cells in cultures infected with primary isolates.** Only one human MAb to the V2 region, MAb 697-D, has been identified to date. The epitope of this MAb appears to span the region from positions 161 to 194 of gp120 (the position numbering is based on HIV<sub>HXB2R</sub> numbering [38]), and it is primarily conformation dependent, with weak reactivity for a linear region between amino acids 161 and 180 (18). This anti-V2 MAb reacted sporadically with viruses from clades B, B', C, and D (Fig. 1). Although no consistent sequence which differentiated the V2 sequences of those viruses that generated cells that were stained with 697-D from those viruses that did not (data not shown) could be identified, a particularly frequent variation was noted in the V2 region from positions 164 to 172 (SIRGKVQKE), a region which was previously noted to be involved in the epitope of MAb 697-D (18). In addition, a correlation was observed (Fig. 1) for those cultures which stained with the anti-CD4bd MAbs. This was confirmed by cluster analysis (see below).

**Expression of CD4bd on cells infected with primary isolates.** As noted above, the staining of cells in infected cultures with anti-CD4bd MAbs was sporadic because of the blockade of the CD4bd by CD4; only infected CD4-negative cells stained with anti-CD4bd MAbs. The staining, when it did occur, was found on cells infected with viruses from clades A, B, C, and D. The data are consistent with previously published results that the CD4bd may be broadly distributed among HIV-1 viruses (27, 35); however, immunofluorescence does not appear to be an

TABLE 2. Correlation of staining with anti-V3 MABs and V3 sequences of isolates used to infect PBMCs

Virus designation	V3 sequence	Cells (%) staining with MAB:				Sequence in virus corresponding to core epitope of MAB
		257-D	268-D	447-52D	694/98D	
US4	KSIHI---GPGRAP	41	42	41	28	KSIHI HIGPGR GPGR GRAF
BZ167	RRIRI---GPGRTF			43	19	GPGR GRTF
B2THO14W	KSIHL---GPGRAP	17	16	18	13	KSIHL HIGPGR GPGR GRAF
TH130	KRIHI---GPGRAP	43	44	44	23	KRIHI HIGPGR GPGR GRAF
US1	KSIHI---GPGRAP	78	78	79	77	KSIHI HIGPGR GPGR GRAI
US2	KSIHI---GPGRAP	73	74	64	43	KSIHI HIGPGR GPGR GRAF
B2US714D	RSIHM---GPGRAP		15	16	17	HMGPR GPGR GRAF
B2HA594D	KRISI---GPGRAS	28		35	23	KRISI GPGR GRAS
B2HA593D	KRISI---GPGRAP	21		24	28	KRISI GPGR GRAF
B2US657D	KGIHI---GPGRAP	39	40	40	28	KGIHI HIGPGR GPGR GRAF
B2US727D	KSVHI---GPGRAP	12	23	14		KSVHI HIGPGR GPGR GRAF
LAI	KSIIRI-QRGPGRAP			19	58	
B2TH026W	KSIPL---GPGQAW					
B2HA599D	RSVHSGHIGGRTL					
TH237	KSIHL---GPGKAW	28				KSIHL

optimal technique for assessing its distribution among the various clades.

**Cluster analysis of the data.** The results of the cluster analysis were utilized to construct a phenogram (not shown) which describes the history of the clustering process and suggests the existence of immunologic relationships as a result of the reactivities of the cells from infected cultures with the MABs specific for various epitopes. This analysis suggests the following clusters.

(i) The anti-gp41 MAB and anti-C terminus MAB 858-D cluster together. (Analysis of anti-C terminus MAB 670-D could not be performed because of an incomplete data set with this MAB; inspection of Fig. 1, however, suggests that MAB 670-D belongs in this cluster.) On the basis of the ubiquity of these epitopes among the various clades, this cluster appears to reflect an immunologic association that identifies the relevant epitopes as being group specific.

(ii) The V3 MABs tend to cluster together among themselves and with 989-D, an anti-C terminus MAB. On the basis of the restricted reactivity of this cluster, it appears to reflect the immunologic association which identifies the relevant epitopes as clade related. The two most closely related pairs of MABs in this cluster are MABs 447-52D and 694/98-D and MABs 257-D and 268-D. These relationships confirm the overlap noted in the mapping of the core epitopes of these MABs (Table 2).

(iii) The anti-CD4bd MABs cluster together and with the anti-V2 MAB. This finding supports the notion of a relationship between the V2 loop and the CD4bd on both immunochemical and functional bases. This relationship has been described previously (14, 34, 46).

## DISCUSSION

The serotyping of infectious organisms is the classical technique used to categorize of organisms within a given genus or species. This approach allows the differentiation of strains of bacteria and viruses on the basis of antigenic structures which are recognized with specific antisera or MABs and provides information on shared immunologic and structural characteristics. Thus, for example, poliovirus is divided into serotypes primarily on the basis of epitopes located on the three structural proteins making up the viral surface (13, 42, 52). Similar immunologic, or serotypic, analyses have been used to categorize most virus families, subfamilies, and genera (13). More

recently, with the development of techniques in molecular biology, serotypic analyses of many bacteria and viruses have been augmented with genotypic classification wherein organisms are categorized by restriction endonuclease mapping or by sequence analysis at the nucleotide level and/or amino acid level.

Like most viruses, HIV-1 and HIV-2 were first distinguished by serologic methods. Thus, Barin et al. (3) and Clavel et al. (8) described sera from West African subjects which reacted more strongly with simian immunodeficiency virus than with HIV-1. Virus isolation and sequence analysis later revealed that this West African virus, HIV-2, was distinct from HIV-1 and that these two viruses represented two different species within the lentivirus genus (21).

In contrast to the typing of most viruses, subclassification of HIV-1 was initially based on nucleotide sequence analysis rather than on serotyping. More than six HIV-1 subtypes, or clades, have now been definitively identified, and they are approximately equidistant from one another on a coding sequence tree (38). Viruses of each clade tend to cluster in geographic areas.

In order to determine how these viruses were immunologically related, whether serotypes exist, and if so, whether serotypes parallel the genotypic groupings, we studied the reactivity of antigens expressed on the surfaces of PBMCs cultured with primary isolates from each of five different genotypes (clades A to E). Flow cytometry was used, as opposed to studying the antigens detected in detergent-treated lysates of virions by immunochemical techniques, to examine the antigenic structure of oligomeric viral glycoproteins in their native configuration on the surfaces of cells rather than partially denatured monomeric glycoproteins captured onto a plastic substrate. Moreover, in order to analyze these antigens in the context of the human anti-HIV immune response, MABs derived from the cells of HIV-1-infected humans were used.

To standardize the method, MABs were first tested for their ability to stain cells infected with a PBMC-adapted laboratory isolate, HIV<sub>LAI</sub>; all of the MABs to the HIV-1 glycoproteins which had previously been shown to react immunochemically with recombinant proteins and peptides of HIV<sub>LAI</sub> were able to stain cells infected with this virus. Interestingly, the patterns of the staining of cells infected with HIV<sub>LAI</sub> and with clade B primary isolates were strikingly similar (Fig. 1 to 3).

The method used, acute infection of stimulated PBMCs with free virus, gave excellent levels of infected cells expressing viral

antigens. The mean percentage of cells stained with the most sensitive and broadly reactive MABs, the two group-specific anti-C terminus MABs, was 36%. While the range of percent positive cells in cultures stained with these two MABs extended from 8 to 82%, the level of infection was consistent with those in studies in the literature showing that 50 to 100% of transformed cells infected *in vitro* are infected (5, 23, 29, 55) and that ~30% of acutely infected PBMCs *in vitro* are infected (23). These figures are strikingly higher than those for the level of infection achieved by cocultivation of activated cells from normal and infected donors, which results in a much lower level of infection that peaks much later (40, 57).

Analysis of the immunologic nature of HIV-1 envelope glycoproteins in their native configuration on the surfaces of infected cells revealed various categories of epitopes.

Group-specific epitopes appear on cells infected with the majority of HIV-1 isolates, regardless of clade. Included in this category are an immunodominant epitope of gp41 located between amino acid residues 579 and 613 and recognized by MAb 50-69 (17, 56) and two distinct antigens at the C terminus of gp120 recognized by MABs 670-D and 858-D. Previous studies have suggested that the immunodominant antigen on gp41 is partially obscured by its association with gp120 (48-50); this appears to be confirmed here on the basis of the lower level of fluorescence obtained with this MAB compared with that for MABs that stained other envelope epitopes.

Clade-restricted epitopes appear on cells in cultures infected with only some or one of the HIV-1 clades. Surprisingly, there appears to be a clade B-restricted epitope in the C terminus of gp120 which is recognized by MAB 989-D. Clade-specific epitopes in this region have not, heretofore, been described, although Blomberg et al. (4) showed that African sera reacted preferentially with peptides from the C terminus of gp120 of two Zairian viruses. While the MAB that recognizes the clade-specific C terminus epitope maps to the same peptide to which the group-specific anti-C terminus MAB 858-D maps (VVQREKR), the two MABs clearly differ in the patterns of viruses they recognize (Fig. 1). Since the specificity of MABs to linear determinants is determined in large measure by conformation (16), this unusual epitope may be primarily conformational, thus providing an explanation for the absence of information about this determinant from sequence analysis. Studies to delineate the different epitopes of several anti-C terminus MABs are continuing.

The other category of clade-restricted epitopes was recognized by anti-V3 MABs. Two anti-V3 MABs recognized cells in cultures infected with viruses of clade B and with viruses of clade A or clade B' (those with aberrant V3 loops compared with those of most clade B viruses). Two other anti-V3 MABs reacted with cells in cultures infected with only clade B viruses, suggesting the existence of a category of clade-specific anti-V3 epitopes. Since V3 is one of the principal neutralizing determinants in primary isolates (9), the data herein support the concept that the V3 loop epitope is important in defining a functionally relevant (neutralizing) serotype. The various anti-V3 MABs may be useful not only in distinguishing between clades but in making distinctions within clades. Thus, while only three clade B' viruses were available for testing, these were distinguished from the clade B viruses by the anti-V3 MABs. Such intratypic subgroups have previously been reported for clade F, as determined by genotypic methods (31). The immunologic data presented herein suggest that at least one intratypic subgroup within clade B may now be discerned immunologically.

It is noteworthy that these various anti-V3 MABs react broadly with clade B virus-infected cultures despite the hyper-

variability of the V3 loop (Table 2). The reactivity of the four anti-V3 MABs in 86% of the analyses with 11 clade B primary viruses suggests the existence of an immunologic similarity between diverse isolates even in this variable region. Human MABs which were stimulated by natural infection as opposed to MABs induced by peptides may be particularly adept at recognizing the conserved shape of the clade B V3 loop. Indeed, all of these human anti-V3 MABs have been shown to bind more avidly to rgp120 than to V3 peptides (16, 20), demonstrating a required conformational aspect of the antigen in order for binding to be maximal.

A third category of epitopes can be detected only sporadically on cells in cultures infected with isolates from the various clades. The best example of this type of epitope is that recognized by the anti-V2 MAB 697-D on cells in cultures infected with a minority of viruses from clades B, B', C, and D. The V2 epitope was expressed on both CD4-positive and CD4-negative cells (data not shown) in 30% of the analyses performed with cultures infected with primary isolates. Similarly, anti-CD4bd MABs stained cells infected by a minority of viruses from clades A to D, staining cells in 23% of the analyses performed. When cells stained with anti-CD4bd MABs were analyzed, it was found that only cells that failed to stain for CD4 with Leu-3a stained with the anti-CD4bd MABs. Interestingly, a relationship between the presence of the V2 and CD4bd epitopes appears to exist, and this relationship can be seen by an inspection of the data (Fig. 1) and by cluster analysis. Evidence for functional and immunochemical relationships between V1/V2 and the C4 region and CD4bd of gp120 has previously been described (14, 36, 46).

The V2 and CD4bd epitopes may be variant specific, i.e., present on cells in cultures infected with only a minority of viruses which, however, are not restricted by membership in any particular clade. Alternatively, one or both of these epitopes may be more widespread but poorly detected on the surfaces of infected cells because of the blockade of the epitopes by gp120 (15). At a minimum, one can state that shared antigens of the CD4bd are found among most of the clades (35), but the degree to which they are shared by individual viruses cannot be ascertained by staining infected PBMCs. Previous studies of virus neutralization (2, 16, 24, 39, 44, 51) showed that only a portion of viruses studied can be neutralized by anti-CD4bd MABs, suggesting that, on this functional criterion, some antigens of this large and complex epitope may fall into the variant-specific rather than the group-specific category.

Quantitative rather than qualitative analysis of the data highlights additional issues. Thus, each of the MABs tested stained cells in infected cultures to various degrees. For example, the anti-C terminus MAB 670-D stained all infected cultures tested, but the level of staining varied from 19 to 82% (Fig. 1). The level of staining with the anti-gp41 MAB 50-69 ranged from 2.2 to 27.1%. This range in the staining by a single MAB of cells infected with different viruses and the differences noted in staining by different MABs of cells infected with a single virus may be due to several nonexclusive factors: the nonclonal nature of the infecting viruses, differences in the affinities of given MABs for any given virus glycoprotein, the various states of glycoprotein processing and glycosylation on the surfaces of infected cells, and the degree to which different primary viruses shed monomeric gp120 which may bind to the surfaces of uninfected cells.

Definition of group-specific and clade-specific antigens and a panel of MABs that would define each will be useful in epidemiologic studies and in some clinical settings, particularly in countries where infection is caused by multiple clades. With



these reagents, definition of the dominant clades causing infection in a defined geographic region or in an individual could be performed relatively simply by fluorescence microscopy, and the need for isolation, sequencing, and/or production of the relatively large amounts of virus needed for immunochromatological analysis could be avoided (35). The MAbs to group-specific antigens would serve as positive controls; the clade-specific MAbs would distinguish between clades. Given the current assumption that reagents for active and/or passive immunization will need to match the locally dominant virus (or the virus in an infected pregnant mother), readily available information about the serotypes of HIV-1 in populations and in individuals is essential.

To date, the vast majority of anti-HIV-1 human MAbs have been derived from PBMCs from North American and European HIV-infected individuals and selected on proteins and peptides of clade B viruses. These have yielded reagents that are useful in categorizing group-specific epitopes, clade-restricted epitopes (which react with clade A-, B-, and B'-infected cells), and clade-specific epitopes (which react exclusively with clade B-infected cells). Studies are now under way to develop MAbs derived from the cells of individuals infected with non-B clades in order to complete a panel of MAbs for serotyping which will contain clade-specific reagents reactive with each of the individual clades of HIV-1. These reagents and the methodology described in this paper provide a practical approach for the serotyping and categorizing of strains of HIV-1 without the sequencing or growing of large quantities of the virus.

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ISOLATE	SUBTYPE	PHENO- TYPE	71:21 (%)	81:66 (%)	87:6 (C-TERM)	88:2 (C-TERM)	88:9 (C-TERM)	95:9 (%)	98:9 (%)	147:57-D (U)	88:49-D (U)	58:49-D (U)	35:5 (C-46)	72:2 (C-46)	88:9 (%)
E27H021W	A	NSI	21	21	42.2	35.9	11.9	2	18	2	1.2	2.3	2.3	4.1	2.3
E27H021W	A	NSI	22	15	N.D.	44	12	38	25	3.1	6.2	3.3	1.2	2.6	2.3
E27H021W	A	N.D.	23	12.2	49.6	33.3	12.7	46.1	1.7	3.3	15.2	2.2	2.9	4.7	2.6
USA	B	NSI	29	14.9	54.8	45.3	26.5	41	41.5	41.1	28	1	3.8	9	3.8
E27H014W	B	NSI	22	16.9	50.4	31.2	11.3	44	3.5	42.3	18.4	2.2	10.1	4.2	14.8
E27H014W	B	NSI	23	17	N.D.	14.9	12	18.5	15.8	17.6	12.8	3	2.9	3.9	2.1
E27H014W	B	NSI	24	12.8	N.D.	37	12.4	43	4.4	45.5	28	2.8	3.4	6.8	3.1
U51	B	NSI	25	12.8	74.8	35	21.3	78.6	77.8	75.2	78.6	1.5	8.3	1.4	68.7
U52	B	NSI	26	12.8	82.1	28.8	16.6	75	73.9	69.8	40	4.3	3.5	6.2	5
E27H021W	B	NSI	27	12.8	41.9	30.1	21.4	4	19.9	19.2	17.2	3.5	3.4	6.2	5.7
E27H021W	B	NSI	28	12.8	58.8	15.3	14	25.2	3.8	31.8	17.2	3.8	3.2	N.D.	2.8
E27H021W	B	NSI	29	11.4	32	29.3	20.1	21.2	2.8	24.4	21.6	0.3	1.9	3.1	3.2
E27H021W	B	N.D.	30	12.8	44.4	3	27	36.3	39.7	39.8	27.6	1.5	1.8	3.1	2.8
E27H021W	B	N.D.	31	12.8	37.2	1.6	34	12.3	53	13.8	5.2	1.2	1.3	2.3	1.3
E27H021W	B	NSI	29	20.1	82.2	57.6	48.3	2.8	2.3	18.1	33.4	7	7.2	6	6.2
E27H021W	B	NSI	23	5	N.D.	4.2	15	27	1.8	1.7	2.4	2.3	2	3.1	2.8
E27H021W	B	NSI	24	7.4	45	24.3	12.8	27	2.5	6.1	1.8	2.3	1.8	3.6	7.4
E27H021W	B	NSI	25	15.6	40.1	54.2	15.3	23.8	3.3	4.2	8.2	2.8	3.3	1.3	2.3
SMH5	C	NSI	18	5.8	N.D.	31	2.7	2.8	2	3.3	2.9	3.2	3.5	4.7	3.8
SG34	C	NSI	27	20.1	71.7	54	3	2.8	5	6.7	4.7	2.2	7.2	11.3	7.6
SG34	C	N.D.	27	4.8	25.3	3.4	2.3	2.8	2.2	2.4	2.7	2.2	2.8	2.7	2
E27H021W	D	NSI	23	6.8	N.D.	18.3	3.8	2	2	2	2.3	7	8.5	1.8	8.5
E27H021W	D	NSI	27	16.3	N.D.	20	2.8	2.1	2	1.8	2.9	2.7	5.3	3.4	1.2
E27H021W	D	NSI	17	2.8	35	13.2	3	4.9	4.4	1.8	2.3	3.1	3.1	3.8	4.5
E27H021W	D	N.D.	21	4.8	31.7	6.8	2.2	3	2	5.1	3.1	3.7	4.7	4.2	7
E27H021W	E	NSI	12	2.2	18.7	2	1.3	1.2	1.3	1.3	1.1	1.1	1.1	1.4	1.4
E27H021W	E	NSI	25	15.3	79.8	13.2	4.1	8.8	12	4.7	1.8	4.3	4.3	5.8	5
E27H021W	E	NSI	18	4.9	20.2	2.3	1.1	1.1	1.2	1	1.1	1.2	1.1	2.2	1.3
NCNE	-	-	27	2.2	2.8	2	2	2.8	2.4	2.4	2.8	2.3	2.1	3.1	2.3

## The Cell Death-Inducing Ability of Glycoprotein 120 from Different HIV Strains Correlates with Their Ability to Induce CD4 Lateral Association with CD95 on CD4<sup>+</sup> T Cells

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### ABSTRACT

CD4 cross-linking by HIV gp120 triggers CD4<sup>+</sup> T cell death. Several authors have suggested that this effect is mediated by CD95, but this possibility is debated by other authors. In a previous work, we found by co-capping that gp120<sub>451</sub> and gp120<sub>MN</sub>, but not gp120<sub>IIB</sub>, induce lateral association of CD4 with CD95 on the T cell surface. In this work, we used fluorescence resonance energy transfer to confirm that CD4/CD95 lateral association is induced by gp120<sub>451</sub>, but not gp120<sub>IIB</sub>. Moreover, we found that gp120 ability to induce the CD4/CD95 association correlates with ability to induce cell death, since gp120<sub>451</sub> and gp120<sub>MN</sub> induced higher levels of cell death than did gp120<sub>IIB</sub> in PHA-derived CD4<sup>+</sup> T cell lines. CD95 involvement in gp120-induced cell death was confirmed by showing that gp120<sub>451</sub> and gp120<sub>MN</sub> did not induce death in CD4<sup>+</sup> T cells derived from patients with autoimmune/lymphoproliferative disease (ALD) and decreased CD95 function. Cell death induced by gp120<sub>MN</sub> was inhibited by a recombinant CD95/IgG.Fc molecule blocking the CD95/CD95L interaction. However, inhibition was late and only partial. These data suggest that the gp120-induced CD4/CD95 association exerts a dual effect: an early effect that is independent of CD95L and may be due to direct triggering of CD95 by gp120, and a late effect that may be due to sensitization of CD95 to triggering by CD95L. In line with the former effect, cell treatment with gp120<sub>MN</sub> activated caspase 3 in the presence of Fas/IgG.Fc, which shows that cell death induced by gp120<sub>MN</sub> independently of CD95L uses the same pathway as CD95.

### INTRODUCTION

THE FIRST STEP FOR CD4<sup>+</sup> T cell infection by HIV-1 involves interaction between the viral envelope glycoprotein gp120 and its main cell surface receptor, CD4.<sup>1,2</sup> gp120 may make other contributions to the immunopathogenesis of AIDS. Soluble gp120 released by infected cells potentiates CD4<sup>+</sup> cell depletion by triggering negative signals via CD4 and inducing lymphocyte anergy or programmed cell death. Moreover, it targets gp120-specific antibodies and cytotoxic cells against uninfected CD4<sup>+</sup> cells presenting unprocessed and processed gp120 on their surface.

We have previously shown that CD4 displays lateral association with several molecules on the T cell surface and that gp120 binding to CD4 alters its lateral association pattern.<sup>3,4</sup> Moreover, gp120 derived from four HIV-1 strains and displaying different primary structure induced partially different CD4 lateral association patterns. All gp120 (i.e., gp120<sub>451</sub>, gp120<sub>IIB</sub>, gp120<sub>SP2</sub>, and gp120<sub>MN</sub>) induced CD4 association with CD3, CD26, CD38, CD45, and CD59, whereas they displayed different abilities to induce association with CD11a, CD27, CD49d, and CD95. Since gp120 is highly variable from one strain to another, we suggested that CD4-bound gp120 interacts with low affinity with other surface molecules and that

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gp120 variability influences the pattern of interactions. Since these molecules are involved in crucial cell functions such as activation/inactivation and adhesion, we suggested that gp120-driven assembly of these abnormal multimolecular complexes could be responsible for gp120-induced CD4<sup>+</sup> T cell dysfunctions. In line with this possibility, we showed that treatment with gp120 alters T cell adhesiveness *in vitro* and homing *in vivo* by altering the adhesive function of CD11a, CD49d, and CD38.<sup>5</sup>

This article compares the ability of different gp120 to induce CD4<sup>+</sup> T cell death. Our starting point was the observation that they differentially induce CD4 association with CD95 (Fas/Apo-1), which is a transmembrane molecule belonging to the tumor necrosis factor (TNF) receptor superfamily and interacts with the CD95 ligand (CD95L), a type II transmembrane molecule belonging to the TNF cytokine superfamily. CD95 ligation by monoclonal antibodies (MAbs) or CD95L induces programmed cell death in several lymphoid cell lines and long term-activated peripheral T cells. In contrast, it mediates costimulation in resting and recently activated T cells.<sup>6,7</sup> The ability of CD95 to induce cell death depends on its connection with a death-signaling pathway inducing activation of several cysteine proteases named *caspases*.<sup>8-10</sup> Connection is mediated by the adaptor molecule FADD/MORT1, which associates with the CD95 cytoplasmic portion and with caspase 8/FLICE. Association with FADD activates caspase 8, which turns on a cascade composed of caspase 10, 6, 2, and 3, and consequently activates the apoptotic machinery. By turning off activated lymphocytes, CD95 plays a role in immune response control and lymphocyte life span regulation. CD4 triggering by gp120 or MAb either induces cell death or "primes" cells for cell death induced by triggering of the CD3/T cell receptor (TCR) complex.<sup>11</sup> Both effects seem to be mediated by the CD95/CD95L system, on which CD4 triggering exerts a dual effect: (1) it sensitizes the cell death-inducing function of CD95 and (2) it induces CD95L expression on type 1 helper T (Th1) cells and macrophages, which will trigger the death of cells expressing the sensitized CD95.<sup>12-20</sup>

Our results show that gp120s from different HIV-1 strains display a different capacity to induce cell death, and that this correlates with their ability to induce CD95/CD4 lateral association on the T cell surface.

## MATERIALS AND METHODS

### Cell purification and immunofluorescence analysis

Peripheral blood (PB) mononuclear cells were prepared by gradient centrifugation (Lymphoprep; Nycomed, Oslo, Norway) of buffy coats obtained from the local blood bank. CD4<sup>+</sup> T cell purification was performed by removing monocytes by plastic adherence and panning with anti-CD8, -CD11b, and -HLA class II MAbs, which remove CD8<sup>+</sup> T cells (anti-CD8 and -CD11b), natural killer (NK) cells (anti-CD11b), and B cells (anti-HLA class II). CD4<sup>+</sup> T cell purity was >85%, as assessed by direct immunofluorescence using the fluorescein isothiocyanate (FITC)-conjugated Leu3 MAb (anti-CD4) (Becton Dickinson, Mountain View, CA) and cytofluorimetric analysis. These cells were cultured for 4–6 days in RPMI–10% fetal calf serum (FCS)–phytohemagglutinin (PHA, 1 µg/ml) and used in the physical association and cell death assays. Af-

ter culture, CD4<sup>+</sup> T cell purity was higher than 95%. Patients with autoimmune/lymphoproliferative disease (ALD) were those previously reported.<sup>21</sup>

Expression of CD95 and CD95L was evaluated with FITC-conjugated anti-CD95 (Chemicon, Temecula, CA) and anti-CD95L (Alexis, San Diego, CA) MAbs. Samples were analyzed with a FACScan flow cytometer (Becton Dickinson). Nonspecific background fluorescence was established with the appropriate isotype-matched control MAb (Becton Dickinson). Antigenic density was expressed as the median fluorescence intensity ratio (MFI-R) of total lymphocytes according to the following formula:

$$\text{MFI-R} = \frac{\text{MFI of sample histogram (arbitrary units)}}{\text{MFI of control histogram (arbitrary units)}}$$

### Cell death assays

PB CD4<sup>+</sup> T cells (10<sup>5</sup> cells/well), cultured for 6 days with PHA–interleukin 2 (IL-2), were incubated with control medium, anti-CD95 MAb (CH11, IgM isotype, 1 µg/ml; UBI, Lake Placid, NY), methylprednisolone (PDN, 100 µM; Upjohn, Puurs, Belgium), recombinant gp120<sub>IIIb</sub> (Intracell, London, UK), recombinant gp120<sub>MN</sub> (Agmed, Bedford, MA), or affinity-purified gp120<sub>451</sub> (ABI, Columbia, MD) (2 µg/ml each) in 96-well plates in a final volume of 200 µl of RPMI–5% FCS–IL-2 (2.5 U/ml). After 24, 48, and 72 hr, cell survival was assessed by counting live cells in each well by the trypan blue exclusion test, as previously reported.<sup>21</sup> Results were expressed as relative cell survival (%), calculated as follows:

$$\frac{(\text{total live cell count in the assay well})}{(\text{total live cell count in the time-matched control well})} \times 100$$

This technique was chosen in preliminary experiments comparing the sensitivity of several techniques to detect cell death triggered by anti-CD95 MAb on PHA-derived T cell lines. It evaluates the overall cell survival in the culture at each time and was found to be more sensitive than techniques detecting the instantaneous proportion of dying cells at each time, such as cytofluorimetric determination of cells displaying shrunken/hypergranular morphology, those displaying DNA fragmentation after staining with propidium iodide, or those stained by annexin V (Ref. 21, and our personal observation, 1998). CD95-induced cell death was always less striking in these polyclonal T cell lines than in stabilized tumor cell lines, since it was slower and more asynchronous. In some experiments, the CD95/CD95L interaction was inhibited by adding recombinant Fas/IgG.Fc (2 µg/ml) (rhAPO-1/Fas:Fc-IgG.Fc kit; Alexis, Läufelfinger, Switzerland) at the beginning of the cell death assay.

In three experiments, the gp120-induced cell death was detected with an Annexin-V-Fluos kit (Boehringer GmbH, Mannheim, Germany). Briefly, 10<sup>6</sup> cells were stained with annexin V (1:50) and propidium iodide (1 µg/ml) in 10 mM HEPES–NaOH (pH 7.4)–140 mM NaCl–5 mM CaCl<sub>2</sub> and analyzed by flow cytometry.

Caspase 3 activity was assessed with an ApoAlert CPP32/caspase 3 fluorescent assay kit (Clontech, Palo Alto, CA) on 2 × 10<sup>6</sup> cells/sample. Results were expressed as a ratio between the caspase activity displayed by cells treated with the indicated reagent and that displayed by untreated cells and were calculated as follows:

Caspase 3 activity ratio =

$$\frac{\text{activity (in fluorescence units) displayed by gp-120 treated cells - background}}{\text{activity (in fluorescence units) displayed by untreated cells - background}}$$

Background was the activity detected in the absence of substrate and ranged from 2 to 5 fluorescence units.

#### Physical association analysis

PB CD4<sup>+</sup> T cells were cultured for 1 day in RPMI-10% FCS-PHA (1 µg/ml), and then washed and cultured for 3 days

in RPMI-10% FCS-recombinant IL-2 (5 U/ml). They were then washed, resuspended in medium (RPMI-10% FCS), and treated with a saturating concentration (2 µg/ml) of gp120<sub>IIIB</sub> or gp120<sub>451</sub>, or with an equal volume of PBS (control cells) at 37°C for 30 min. Cells were then moved to ice and used in the fluorescence resonance energy transfer (FRET) assay. The Cy3-conjugated OKT4 (CD4) MAb and FITC-conjugated anti-CD95, -CD25, and -CD7 MABs (Becton Dickinson) were used. OKT4 conjugation with the Cy3 dye was performed with a FluoroLink-Ab Cy3 labeling kit (Amersham, Arlington Heights, IL). Cells were washed with ice-cold PBS-5% FCS-0.1% NaN<sub>3</sub> and incubated on ice for 1 hr simultaneously with the FITC-conjugated MAB and the Cy3-conjugated OKT4. They were

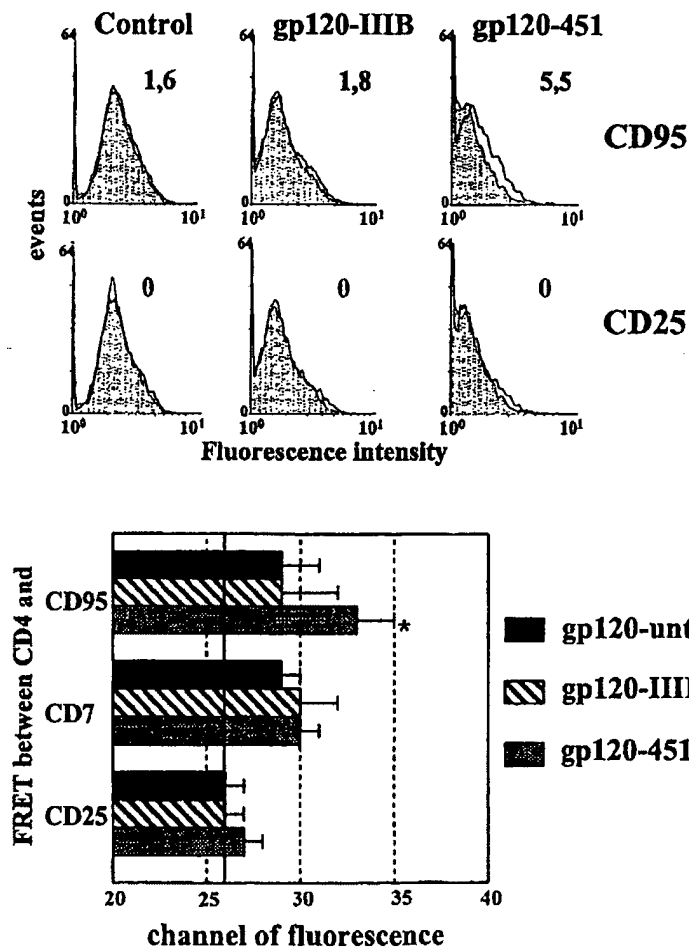


FIG. 1. gp120<sub>IIIB</sub> and gp120<sub>451</sub> induce different association levels of CD4 with CD95 in PHA-activated CD4<sup>+</sup> T cells. FRET between CD4 and CD95 is significantly higher in cells treated with gp120<sub>451</sub> than in those treated with gp120<sub>IIIB</sub>. In contrast, treatment with any gp120 did not alter FRET of CD4 with CD25 or CD7. Cells were stained with Cy3-conjugated OKT4 MAb and the indicated FITC-conjugated MAB. FITC was excited at 488 nm, Cy3 emissions were collected at >600 nm, and the median fluorescent channel was evaluated. The FACS profiles show representative data from five experiments. The gray curves represent the control curves and show Cy3 emissions at >600 nm (FL3) in the presence of an irrelevant isotype-matched (IgG<sub>2b</sub>) FITC-conjugated MAB. The white curves show the Cy3 emissions in the presence of the indicated FITC-conjugated MAB. A right shift of the curve indicates FRET. Numbers in boldface represent the median positive shift, expressed as median fluorescent channels relative to the control. The bar graph shows the means  $\pm$  SD of data from the five experiments. The solid vertical line shows the Cy3 emissions of the control sample. The asterisk (\*) marks data that are significantly different from those obtained with cells treated with gp120<sub>IIIB</sub> (nonparametric Wilcoxon test for paired samples,  $p < 0.05$ ).

then washed, resuspended in PBS-0.1% NaN<sub>3</sub>, and analyzed immediately. A FACScan flow cytometer was used to determine FRET between FITC (the donor fluorophore) and Cy3 (the accepting fluorophore) on the cell surface, using standard methods.<sup>22,23</sup> FITC was excited at 488 nm and Cy3 emissions were collected at >600 nm. Data from 10,000 cells/test were stored in list mode and analyzed with LYSYS II software (Becton Dickinson). Fluorescence intensity was detected as MFI-R, converted in median linear channels of fluorescence, and used as the indicator for the presence (a positive shift over background) or absence (no shift or negative shift over background) of FRET. FL1 was calibrated with unstained cells and FL3 was calibrated with cells stained with Cy3-conjugated OKT4. The control curves were set on the first log of the scale. All experiments were performed in a 2-week period and the instrument

setting was the same in all experiments (i.e., photomultiplier at 629 V for FL1 and at 430 V for FL3). No fluorescence compensation was set.

Cocapping experiments were performed as previously reported,<sup>4</sup> using two sets of MAb: one was arsonate conjugated and stained with affinity-purified FITC-conjugated rabbit anti-arsonate serum, and the other was biotin conjugated and stained with rhodamine (TRITC)-conjugated avidin.

## RESULTS

In a previous work, we compared the ability of gp120 from four HIV-1 strains to induce CD4/CD95 lateral association on

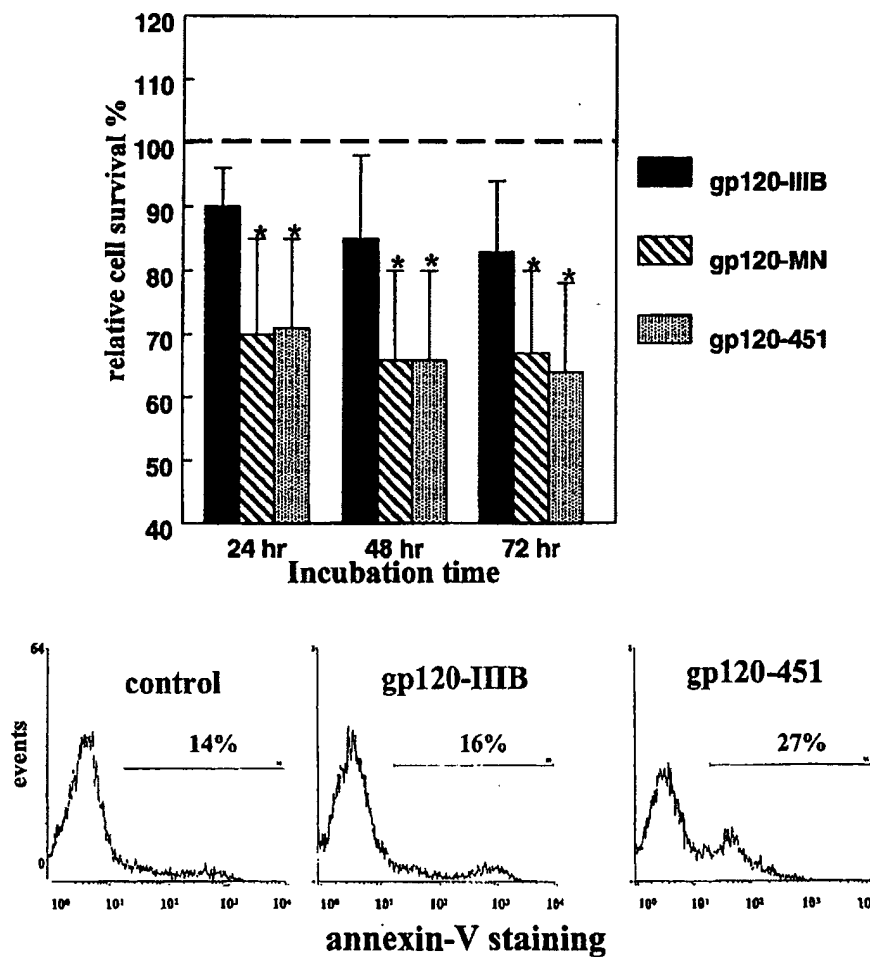


FIG. 2. gp120 derived from different HIV strains induces different levels of death in PHA-activated CD4<sup>+</sup> T cells. *Top*: Cells were treated with the indicated gp120 and cell survival was evaluated after 24, 48, and 72 hr. Results are expressed as relative cell survival (%) and represent the means  $\pm$  SD of 18 experiments. Survival of untreated control cells (100% specific cell survival) is shown by the horizontal dashed line. Asterisks (\*) mark data that are significantly different from those obtained with cells treated with gp120<sub>IIIB</sub> (Wilcoxon test for paired samples,  $p < 0.05$ ). *Bottom*: Cells were treated with the indicated gp120 and cell apoptosis was evaluated after 18 hr by staining with annexin V and propidium iodide. The FACS profiles show staining with annexin V of propidium iodide-negative cells from the untreated and gp120<sub>IIIB</sub>- and gp120<sub>451</sub>-treated samples. One representative experiment of three is shown.

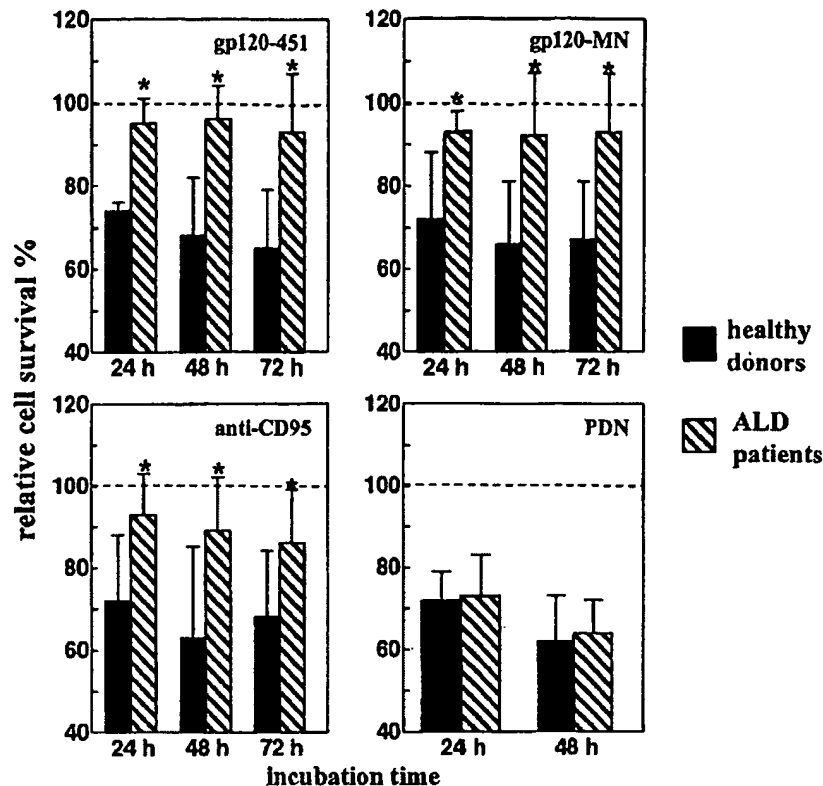


FIG. 3. Cell death induction by several stimuli in PHA-activated CD4<sup>+</sup> T cells from patients with ALD and normal controls (healthy donors). Anti-CD95 MAb, gp120<sub>451</sub>, and gp120<sub>MN</sub> were less able to induce cell death in ALD patients ( $n = 5$ ) than in normal controls ( $n = 16$ ). Cell death induced by PDN was not different in ALD patients and normal controls. PHA-activated CD4<sup>+</sup> T cells were cultured for 24, 48, and 72 hr in the presence of each stimulus and cell survival was then assessed. Results are expressed as Fig. 2. Asterisks (\*) mark data that are significantly different from those obtained with the healthy donors (Wilcoxon test,  $p < 0.05$ ).

the surface of CD4<sup>+</sup> T cells by cocapping.<sup>4</sup> gp120<sub>451</sub> displayed the highest activity, followed by gp120<sub>MN</sub>, whereas gp120<sub>IIIIB</sub> did not induce any association. gp120<sub>SF2</sub> induced an association level that was intermediate between those induced by gp120<sub>MN</sub> and gp120<sub>IIIIB</sub>, but it was not significantly different from any of them. We began this study by performing experiments designed to confirm those results, using the FRET technique. PHA-activated CD4<sup>+</sup> T cells were treated for 30 min with gp120<sub>451</sub> or gp120<sub>IIIIB</sub>, and then they were stained with Cy3-conjugated OKT4 MAb in the presence and absence of a FITC-conjugated anti-CD95 MAb, and FRET between the donor (FITC) and the acceptor (Cy3) fluorochrome was evaluated with a cytofluorimeter. FITC-conjugated anti-CD7 and anti-CD25 MAbs were used as control MAbs carrying the donor fluorochrome. These experiments confirmed the cocapping results, since CD4/CD95 displayed a low basic level of FRET that was significantly potentiated by cell treatment with gp120<sub>451</sub>, but not with gp120<sub>IIIIB</sub>. In contrast, both gp120s had no effect on FRET between CD4 and CD25 or CD7 (Fig. 1). In parallel, we performed cocapping experiments that confirmed the previous results and showed that CD4/CD95 cocapping was significantly higher ( $p < 0.05$ , Wilcoxon test for paired samples) in cells treated with gp120<sub>451</sub> ( $32 \pm 10$ ) or gp120<sub>MN</sub> ( $20 \pm 6$ ) than in untreated cells ( $8 \pm 6$ ) or cells treated with gp120<sub>IIIIB</sub>

( $10 \pm 6$ ) (results are expressed as means  $\pm$  SD of the proportion of CD4-capping cells showing CD95 cocapping). CD4 staining of gp120-treated cells was slightly weaker than that displayed by untreated cells. In contrast, CD95 staining was not affected by cell treatment with gp120. Therefore, our FRET and cocapping assays probably underestimated the gp120-induced CD4/CD95 association because of the inhibition of CD4 staining and we cannot rule out the possibility that gp120<sub>IIIIB</sub> induced low levels of CD4/CD95 association that could not be detected by our assays.

Several authors suggested that the proapoptotic effects of gp120 on CD4<sup>+</sup> T cells is mediated by the CD95/CD95L system.<sup>12-20</sup> Therefore, we evaluated the possibility that the variable gp120 ability to induce the CD4/CD95 association modulates their cell death-inducing capacity. PHA-activated CD4<sup>+</sup> T cells were cultured in the presence and absence of gp120<sub>451</sub>, gp120<sub>MN</sub>, and gp120<sub>IIIIB</sub>, and cell survival was evaluated after 24, 48, and 72 hr. Figure 2 shows that all gp120 significantly induced cell death ( $p < 0.05$ , Wilcoxon test), but gp120<sub>451</sub> and gp120<sub>MN</sub> were significantly more potent than gp120<sub>IIIIB</sub>. Relative cell survival maximally decreased after 24 hr and it apparently reached a plateau at 48 and 72 hr, which was probably due to consumption of gp120. Moreover, the cell lines displayed low levels of proliferation and spontaneous cell death,



which accumulated their effects and partially masked the gp120-induced cell death in the late phases of the assay. To confirm that the gp120 effect was exerted on cell death, we compared the cell death-inducing ability of gp120<sub>IIIB</sub> and gp120<sub>451</sub> by staining apoptotic cells with annexin V. These experiments confirmed that gp120<sub>451</sub> induced higher levels of apoptosis than did gp120<sub>IIIB</sub> (Fig. 2).

To assess involvement of the CD95 system in gp120-induced cell death, we evaluated the ability of gp120<sub>451</sub> and gp120<sub>MN</sub> to induce the death of CD4<sup>+</sup> T cells derived from patients with ALD, carrying defects of the CD95 apoptosis pathway.<sup>21</sup> T cells from these patients express normal levels of CD95 and CD95L, but are resistant to cell death induced by CD95 triggering since CD95 is not functional. These experiments showed that ALD patient CD4<sup>+</sup> T cells resisted cell death induced both by anti-CD95 MAb and these gp120s (Fig. 3). In contrast, they were normally sensitive to cell death induced by PDN, which is not mediated by CD95.

These experiments showed that a functional CD95 is necessary for gp120-induced death. To investigate the role played by the CD95/CD95L interaction, we evaluated the expression level of CD95 and CD95L in the CD4<sup>+</sup> T cell lines treated or not treated with the different gp120. We also evaluated the ability of a soluble form of CD95 (i.e., the recombinant fusion molecule Fas/IgG.Fc) blocking the CD95/CD95L interaction to inhibit gp120-induced cell death. These experiments were performed with gp120<sub>IIIB</sub> and gp120<sub>MN</sub>, both recombinant gp120 being produced in baculovirus. PHA-derived CD4<sup>+</sup> T cell lines were cultured with gp120<sub>IIIB</sub> or gp120<sub>MN</sub> in the presence and absence of Fas/IgG.Fc and cell survival was evaluated after 1, 2, and 3 days. Moreover, at each time, expression of CD95 and CD95L was evaluated with the appropriate FITC-conjugated MAb. These experiments showed that cell treatment with these gp120 molecules did not significantly alter surface expression of either CD95 or CD95L, evaluated both as proportion of pos-

itive cells and as median expression density (data not shown). Fas/IgG.Fc partially inhibited cell death induced by gp120<sub>MN</sub>, detectably on day 2 and significantly only on day 3 (Fig. 4).

CD95 triggering activates a caspase cascade. Therefore, we evaluated the ability of gp120 to activate the late caspase 3. PHA-derived CD4<sup>+</sup> T cells were treated or not treated with gp120<sub>IIIB</sub>, gp120<sub>MN</sub>, or anti-CD95 MAb, and activation of caspase 3 was evaluated after 7 hr. The experiment was performed in the presence and absence of Fas/IgG.Fc blocking of the CD95/CD95L interaction. These experiments showed that cell treatment with gp120<sub>MN</sub>, but not gp120<sub>IIIB</sub>, activates caspase 3. Moreover, the activation induced by gp120<sub>MN</sub> was only partially inhibited by Fas/IgG.Fc, which suggests that caspase 3 activation is partly independent of CD95 triggering by CD95L (Fig. 5).

## DISCUSSION

In AIDS patients, uninfected PB lymphocytes tend to undergo spontaneous apoptosis.<sup>24,25</sup> The observation that CD4 cross-linking by MAb or gp120 "primes" cells for apoptosis induced by subsequent stimulation of the CD3/TCR<sup>11</sup> has directed the attention of several authors to the role played by CD4 triggering in depletion of uninfected CD4<sup>+</sup> cells. Subsequently, CD4 triggering has been shown to induce cell death even in the absence of activating stimuli in certain experimental systems and several lines of evidence showed that the proapoptotic activity of gp120 is mediated by the CD95/CD95L system.<sup>12-20</sup> First, triggering of CD4 by gp120 or anti-CD4 MAb upregulates expression of CD95 and sensitizes the CD95 proapoptotic function in resting CD4<sup>+</sup> cells, whose CD95 is disconnected from the death pathway.<sup>9,10</sup> Second, it upregulates CD95 on CD4<sup>+</sup> T cells and CD95L on Th1 cells and macrophages, and apoptosis induction seems to depend on the presence of these

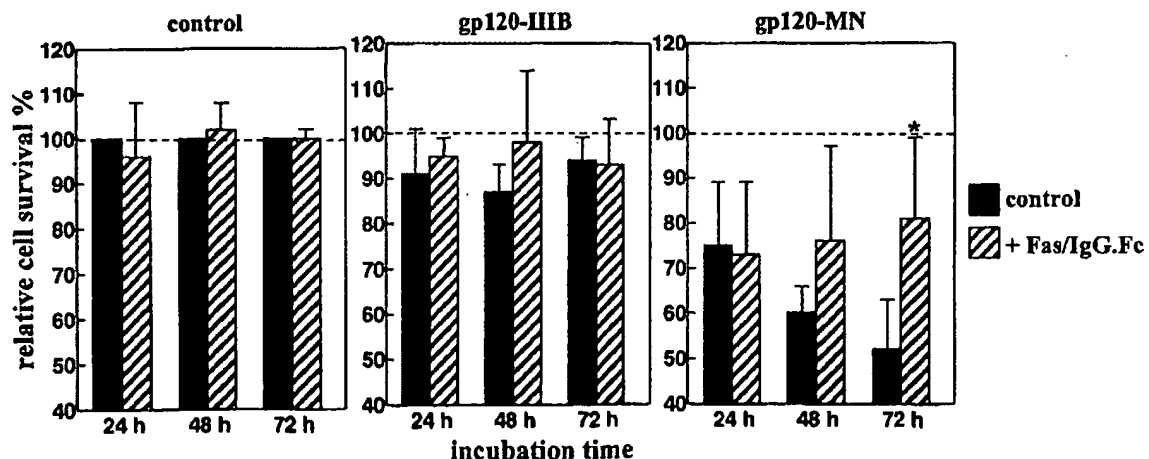


FIG. 4. Effect of recombinant Fas/IgG.Fc blocking of the CD95/CD95L interaction on cell death induced in PHA-activated CD4<sup>+</sup> T cells by gp120. Fas/IgG.Fc partially inhibits the cell death induced by gp120<sub>MN</sub>. The experiments ( $n = 5$ ) were performed as in Fig. 2 and Fas/IgG.Fc was added at the beginning of the culture. Results are expressed as in Fig. 2. The asterisk (\*) marks the data that are significantly different from the relative control (i.e., cells treated in the same manner in the absence of Fas/IgG.Fc) (Wilcoxon test for paired samples,  $p < 0.05$ ).

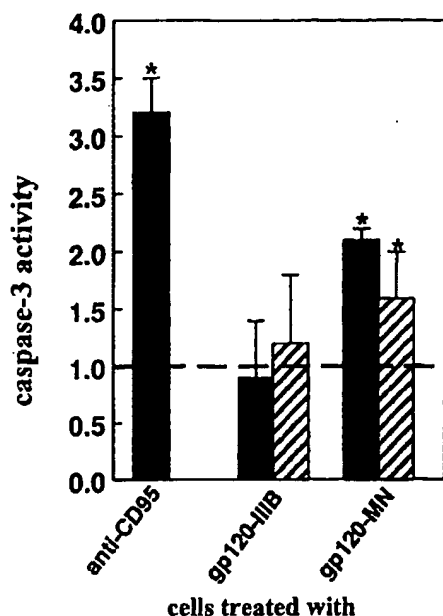


FIG. 5. Activation of caspase 3 in response to anti-CD95 MAb, gp120<sub>MN</sub>, or gp120<sub>IIIIB</sub>. Caspase 3 activity is induced by the anti-CD95 MAb and gp120<sub>MN</sub>, but not gp120<sub>IIIIB</sub>. Fas/IgG.Fc partially inhibits caspase 3 activity induced by gp120<sub>MN</sub>. PHA-derived CD4<sup>+</sup> T cell lines were treated with the indicated reagent in the absence (solid columns) and presence (hatched columns) of Fas/IgG.Fc, and caspase 3 activity was assessed after 7 hr. Data are expressed as the ratio between the caspase activity displayed by cells treated with the indicated reagent and that displayed by untreated cells (shown by the horizontal dashed line) (absolute activity displayed by untreated cells ranged from 10 to 150 fluorescence units). They represent the means  $\pm$  SD of data from four experiments. Asterisks (\*) mark data that are significantly different from those obtained with control cells (Wilcoxon test for paired samples,  $p < 0.05$ ).

CD95L<sup>+</sup> cells in culture. Third, the proapoptotic effect of CD4 triggering is inhibited by soluble forms of CD95 and by anti-CD95 or anti-CD95L MAb blocking the CD95/CD95L interaction. Fourth, CD4 triggering by MAb does not induce cell death in CD4<sup>+</sup> T cells from MLR *lpr/lpr* mice, whose CD95 is nonfunctional owing to mutations of the CD95 gene. However, the role played by the CD95/CD95L interaction is debated by other authors, who have shown that apoptosis does not depend on this system in HIV-infected cell lines.<sup>26-28</sup> These discrepancies may be due to the multiple interactions of HIV with the cell apoptotic machinery, since this involves several HIV products other than gp120 that display pro- or antiapoptotic effects.<sup>12,29-32</sup>

In this work, we show that gp120-induced cell death is strikingly decreased in CD4<sup>+</sup> T cells from ALD patients, who display deficiency of the CD95 apoptosis pathway owing to unknown genetic defect(s) of the CD95 signaling pathway downstream from CD95 and develop chronic autoimmune thrombocytopenia and/or anemia, lymphadenopathy, and/or splenomegaly.<sup>21</sup> This immunopathologic picture resembles that displayed by patients carrying loss-of-function mutations of the

CD95 gene.<sup>33,34</sup> These data show that gp120-induced cell death requires a functional CD95 and confirm those obtained by other authors in *lpr/lpr* mice, which carry loss-of-function mutations of the Fas gene and are resistant to cell death induced by anti-CD4 MAb. However, they do not explain how CD4 triggering recruits the CD95 system. The most obvious possibility is that the effect is mediated by CD4 signaling, which may either up-regulate CD95 and CD95L expression or improve the CD95 connection with the death-signaling pathway.

We suggest a novel mechanism by which gp120 may sensitize the CD95 system, i.e., by inducing the association of CD95 with CD4. gp120 from different HIV strains, in fact, displayed a different ability to induce CD4/CD95 association and this was correlated with their ability to induce cell death. These differences were not dependent on the source of gp120, since gp120<sub>IIIIB</sub> and gp120<sub>MN</sub> were recombinant gp120 prepared from baculovirus and only gp120<sub>451</sub> was affinity purified from infected cells. Moreover, they were not dependent on the gp120 aggregation state, since the same results were obtained with preparations ultracentrifuged to eliminate the aggregates (data not shown). gp120 is highly variable from one strain to another and this variability is probably responsible for its different effects. In our system, the cell death-inducing capacity of the three gp120 was not linked to their ability to upregulate expression of CD95 and CD95L, which were already fully upregulated in our CD4<sup>+</sup> T cell lines. Therefore, gp120 seemed to act mainly by increasing CD95 efficiency in transducing the death signal. Two not mutually exclusive possibilities may be envisaged: (1) CD4/CD95 association sensitizes CD95 responsiveness to CD95L, or (2) it directly triggers the death signal via CD95. In line with the first possibility, we found that cell death induced by gp120<sub>MN</sub> is inhibited by Fas/IgG.Fc blocking of the CD95/CD95L interaction. In line with the second possibility, the blocking effect of Fas/IgG.Fc was only partial and absent at the beginning of the test. Moreover, the observation that cell treatment with gp120<sub>MN</sub> activated caspase 3 under experimental conditions in which the CD95/CD95L interaction was blocked shows that the cell death it induces uses the same pathway as CD95. Therefore gp120 seems to have a dual effect on CD95: an early effect that is independent of CD95L and may be due to direct triggering of CD95 by gp120, and a late effect that may be due to sensitization of CD95 to triggering by CD95L. Both effects might be mediated by CD95 oligomerization driven by the CD4/CD95 association, since oligomerization is crucial for CD95 signaling. CD95 oligomerization may be due to association of several CD95 molecules to the same gp120/CD4 complex, or to the aggregation of several CD4/CD95 complexes driven by interaction with MHC class II-expressing cells. Multivalent gp120 may induce further oligomerization. Alternatively, CD4/CD95 association may potentiate CD95 signaling because of interactions between the signaling machineries of the two molecules. CD95 triggers the death signal via the caspase pathway, which seems to be independent of tyrosine kinases of the Src family.<sup>35-37</sup> However, triggering of CD95 induces tyrosine phosphorylation and activates the tyrosine kinase p56<sup>lck</sup>, which associates with CD4, and p59<sup>fyn</sup>, which associates with the CD3/TCR complex.<sup>38-40</sup> Moreover, Szabo *et al.* reported that p56<sup>lck</sup> is required for the CD95-mediated activation of a chloride channel involved in apoptosis induction.<sup>41</sup> Therefore, p56<sup>lck</sup> is probably not neces-

sary for CD95 function, but it may play a role in the overall function of this molecule and CD4/CD95 association may favor recruitment of the kinase.

Under our experimental conditions, high levels of CD95 and CD95L were expressed since most T cells were activated, which minimized the gp120 effect on CD95 and CD95L expression and maximized that on CD4/CD95 association. Therefore, the cell death-inducing activity of the different gp120s may be different under other experimental conditions or *in vivo*. Moreover, it must be emphasized that these experiments were performed with gp120 derived from X4 HIV strains and no data are available on R5 strains to date.

In conclusion, our data show that gp120-induced cell death requires a functional CD95 and suggest a novel mechanisms by which gp120 may recruit CD95, i.e., induction of CD4/CD95 association. By direct triggering of CD95 or sensitizing of CD95 to its ligand, this association may cooperate with the mechanisms described by other authors in inducing death of CD4<sup>+</sup> cells. The observation that gp120s derived from different viral strains display different capacities to induce the CD4/CD95 association suggests that these mechanisms are used with different efficiency by different gp120s. These differences might play a role in the clinical and biological differences displayed by HIV strains. Rates of AIDS development and CD4<sup>+</sup> T cell depletion are highly variable in HIV-infected individuals and some of this variability might be due to the variable ability of gp120 to interact with the CD95 system. This may account for the finding by Gulizia *et al.*, i.e., that the gp120 gene determines the rate of CD4<sup>+</sup> T cell depletion in HIV-1-infected SCID mice engrafted with human PB leukocytes.<sup>42</sup>

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# Quantification of HIV-1 virus load under zidovudine therapy in patients with symptomatic HIV infection: relation to disease progression

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**Objective:** To measure changes in HIV-1 virus load following zidovudine therapy, and to investigate the relationship between these changes and clinical progression.

**Design:** Prospective study of 18 symptomatic, zidovudine-naïve patients, with CD4 count  $< 350 \times 10^6/l$ .

**Methods:** The following parameters were measured at each visit, before zidovudine therapy, after 1 month of therapy, and every 3 months thereafter. HIV-1 virus load in peripheral blood was determined by serum immune complex-dissociated HIV-1 p24 antigen (ICD-p24 Ag), quantitative plasma and cellular viraemia. A virologic response under zidovudine was defined as  $> 50\%$  decrease in ICD-p24 Ag levels or  $> 1 \log_{10}$  decrease in plasma or cellular viraemia titres from baseline values. CD4 and CD8 cell counts, and  $\beta_2$ -microglobulin levels were also measured. Disease progression was defined as the time to a new AIDS-defining event or death.

**Results:** At enrolment, 13 out of 18 (72%) patients had positive ICD-p24 Ag and positive plasma viraemia, with a mean of 44 median tissue culture infective dose (TCID<sub>50</sub>) per ml; all patients had positive cellular viraemia with a mean TCID<sub>50</sub> of 230 per  $10^6/l$  cells. Median CD4 cell count was  $43 \times 10^6/l$ . Ten patients developed a new AIDS-defining event and eight died during a median follow-up of 15 months on zidovudine. Baseline prognostic markers for development of a new AIDS-defining event included ICD-p24 Ag, CD4 and CD8 cell counts, but only CD4 cell count remained predictive on multivariate analysis ( $P=0.003$ ). When each laboratory marker was analysed as a time-dependent covariate, only CD4 ( $P=0.002$ ) and CD8 ( $P=0.001$ ) cell counts predicted the occurrence of a new AIDS-defining event. Eight out of 13 (61.5%) patients had an ICD-p24 Ag response, and seven out of 13 (54%) a plasma viraemia response, but only cellular viraemia responders (five out of 18; 28%) had a 5.6-fold decrease in their risk of developing an AIDS-defining event (90% confidence interval, 1–33;  $P=0.05$ ). None of these markers correlated with survival.

**Conclusions:** Plasma viraemia and ICD-p24 Ag, while providing useful short-term markers of zidovudine antiviral activity *in vivo*, do not correlate with disease progression in patients with advanced HIV infection. CD4 cell count remained the best initial and time-dependent predictor for development of new AIDS-defining events. Interestingly, a high CD8 cell count and a decrease in cellular viraemia titres also appear to be predictive of improved clinical outcome in this population.

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**Keywords:** HIV, plasma viraemia, cellular viraemia, CD4, CD8, p24 antigen.

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## Introduction

HIV infection is a chronic viral infection with permanent replication of the virus in blood cells and lymphoid organs, characterized by a continuous depletion of CD4 T cells [1]. To date, therapeutic trials have relied on clinical symptoms of disease progression to evaluate the efficacy of antiretroviral drugs. Although these trials have clearly demonstrated the benefit of drugs such as zidovudine, long-term follow-up and large cohorts of patients who will experience clinical deterioration are needed. Moreover, because the number of drugs to be tested in clinical trials is increasing, there is an urgent need to develop surrogate end-points that would reflect antiviral activity *in vivo* and correlate with improved clinical outcome. CD4 cell count is the most widely used prognostic marker for disease progression [2], but as Choi *et al.* [3] have demonstrated recently, it is an incomplete surrogate marker for clinical progression in asymptomatic HIV-infected people taking zidovudine. The limited value of CD4 cell count as a prognostic marker has also been suggested by the preliminary results of the Concorde trial [4].

Plasma viraemia, cellular viraemia and immune complex-dissociated HIV-1 p24 antigen (ICD-p24 Ag) are virological markers of HIV infection that have been shown to increase with advanced stages of disease and decline in response to treatment with antiretroviral agents [5–11]. However, the virological response to antiretroviral therapy has only been evaluated in short-term studies, and has not as yet been shown to correlate with clinical outcome.

In this study, we measured changes in peripheral blood virus load in zidovudine-naïve patients with symptomatic HIV infection following initiation of zidovudine therapy to further investigate the relationship between these changes and clinical progression.

## Patients and methods

### Patients

Between October 1990 and June 1991, 18 HIV-infected zidovudine-naïve patients were prospectively and successively enrolled in this study. All patients had symptomatic HIV infection and a CD4 cell count  $< 350 \times 10^6/l$ . Informed written consent was obtained. Patients began treatment on 200 mg zidovudine three times daily. (In France, zidovudine is recommended for patients with CD4 cells  $< 350 \times 10^6/l$ .) Zidovudine doses were transiently decreased to 300 mg/day in case haematologic adverse effects were encountered. Patients were withdrawn from the study if the drug was discontinued. *Pneumocystis carinii* pneumonia (PCP) pro-

phylaxis was administered in all patients except two with CD4 cells  $> 200 \times 10^6/l$ , using aerosolized pentamidine in nine patients, cotrimoxazole in one, sulphadiazine-pyrimethamine in five with previous cerebral toxoplasmosis, and dapsone in one. Study participants were staged according to the Centers for Disease Control and Prevention (CDC) classification [12]. All patients were monitored by the same physician, who collected clinical data at each visit scheduled after 1 month of zidovudine and every 3 months thereafter. Clinical progression in these patients was defined as the occurrence of a new AIDS-defining event or death. AIDS-defining events were diagnosed according to CDC guidelines [12]. All patients were followed until July 1992, or death.

### Methods

Laboratory tests were performed at each visit. CD4 and CD8 cell counts were determined by flow cytometry and  $\beta_2$ -microglobulin ( $\beta_2M$ ) levels by radioimmunoassay. HIV-1 virus load in peripheral blood was assessed by ICD-p24 Ag and quantitative plasma and cellular viraemia. The consensus method established by the Agence Nationale de Recherche sur le SIDA [13], based on the work by Ho *et al.* [5], and Coombs *et al.* [6], was used to determine cellular and plasma viraemia titres in this study.

### Plasma viraemia

Briefly, 30 ml heparinized venous blood were collected from each patient and assayed within 4 h of collection. Plasma was centrifuged (3000g for 15 min), serially diluted fivefold six times, and added in triplicate wells to the culture medium [RPMI-1640 with 15% fetal calf serum (FCS), 20 IU/ml interleukin-2 (IL-2), penicillin and streptomycin] of  $2 \times 10^6$  fresh phytohaemagglutinin (PHA)-stimulated peripheral blood mononuclear cells (PBMC) from HIV-seronegative donors. Cultures were performed in 24-well plates and maintained for 28 days. Supernatants were assayed for HIV-1 p24 antigen using an immunocapture assay (Abbott Laboratories, Chicago, Illinois, USA) at days 14 and 28. HIV titres were expressed in median tissue culture infective dose (TCID<sub>50</sub>) per ml plasma. TCID<sub>50</sub> were calculated according to the Reed and Muench method [14].

### Cellular viraemia

Six fivefold serial dilutions of cell samples isolated by Ficoll-Hypaque gradient centrifugation were cocultured in triplicate in 24-well plates with  $2 \times 10^6$  fresh PHA-stimulated PBMC from HIV-seronegative donors in RPMI-1640 medium supplemented with 15% FCS, 20 IU/ml IL-2, penicillin and streptomycin. The cocultures were monitored for up to 28 days, and HIV-1 p24 antigen assays were performed at day 14 and 28. HIV titres were expressed as TCID<sub>50</sub>  $\times 10^6/l$  cells [14].

**ICD-p24 Ag**

Serum samples from each patient were collected at each visit and kept frozen at  $-80^{\circ}\text{C}$  until assayed. At the end of the study, all samples were assayed with the same immunocapture kit for ICD-p24 Ag (New Coulter ICD-PREP, Coulter Immunology, Hialeah, Florida, USA) according to the manufacturer's instructions. This assay breaks immune complexes and increases the sensitivity of p24 antigen detection. Serum was considered positive after confirmation with a neutralization assay using polyclonal human anti-HIV-1 immunoglobulin G (IgG). ICD-p24 Ag was quantified in serum using a standard curve generated with serial dilutions of a standard antigen reagent.

A virologic response under zidovudine was defined as  $>50\%$  decrease in ICD-p24 Ag levels, or  $>1 \log_{10}$  decrease in plasma or cellular viraemia titres from baseline values.

**Statistical analysis**

CD4 and CD8 cell counts,  $\beta_2\text{M}$  levels, ICD-p24 Ag, plasma and cellular viraemia measured at months 1, 4, 7 and 10 were compared to baseline values using the Mann-Whitney test. At month 10, 13 patients (72%) were still on follow-up. The Kaplan-Meier method was used to estimate the time to clinical disease progression as described above. The predictive value for disease progression of eight baseline parameters (age, sex, CD4 and CD8 cell counts, cellular viraemia, plasma viraemia, ICD-p24 Ag and  $\beta_2\text{M}$ ) was assessed by log-rank test. Those variables selected at the 5% level by log-rank test were simultaneously introduced into a Cox regression model [15] to summarize prognostic information in a multivariate analysis. Moreover, to explore the relationship between viral load and disease progression, laboratory markers were introduced separately as time-dependent covariates in a Cox regression model. Two-sided tests were performed. Data are provided as mean  $\pm$  SE. Correlations between plasma viraemia, cellular viraemia and ICD-p24 Ag were performed at baseline and under zidovudine therapy using the Spearman's rank coefficient. Statistical analysis was performed using SAS (SAS Institute, Cary, North Carolina, USA) and BMDP packages (University of California, Berkeley, California, USA).

**Results****Baseline clinical characteristics**

Among the 18 patients enrolled in this study, 13 were CDC stage C3 [12], one stage C2, three stage B3 and one stage B2. Therefore, 17 out of 18 (94%) patients had had a previous diagnosis of AIDS before entering the study. Mean age was 33 years, 16 patients were men and two women. Risk factors

for HIV infection were homosexual contact (55.5%), heterosexual contact (22%), intravenous drug use (17%), and blood transfusion (5.5%). One patient was lost to follow-up and censored after 1 month of zidovudine. Mean duration of follow-up under zidovudine was 15 months (range, 1–21 months).

**Immunological markers**

Mean CD4 cell count at baseline was  $97 \times 10^6/\text{l}$  (range,  $0\text{--}350 \times 10^6/\text{l}$ ; median,  $43 \times 10^6/\text{l}$ ). A transient increase of CD4 cell count was noted at 1 month [mean,  $128 \times 10^6/\text{l}$ ;  $P=0.06$ ; median,  $80 \times 10^6/\text{l}$  (Fig. 1a)]. Mean CD8 cell count at baseline was  $809 \times 10^6/\text{l}$  (range,  $150\text{--}1628 \times 10^6/\text{l}$ ; median,  $678 \times 10^6/\text{l}$ ) and did not change significantly during follow-up. Mean  $\beta_2\text{M}$  level was 4.1 mg/l at baseline, and a transient but statistically significant decrease of 3.3 mg/l ( $P=0.02$ ; data not shown) was observed at 1 month, but not thereafter.

**Virus load markers**

ICD-p24 Ag was detected in 13 out of 18 (72%) patients at baseline with a mean of  $679 \pm 393 \text{ pg/ml}$ . Eight of 13 patients (61.5%) with detectable antigenaemia had  $>50\%$  decrease in ICD-p24 Ag after 1 month of therapy. Five severely immunosuppressed patients (CD4 count  $<96 \times 10^6/\text{l}$ ) had negative ICD-p24 Ag at baseline and remained negative during follow-up. Two of these patients were of African origin, a population with a low prevalence of p24 antigenaemia. A transient non-significant decrease in ICD-p24 Ag was observed during the first 7 months of zidovudine therapy (Fig. 1b).

Plasma viraemia was also detected in 13 out of 18 (72%) patients at baseline with mean TCID<sub>50</sub> of  $44 \pm 19$  per ml. Three other patients had detectable plasma viraemia during follow-up, so 16 (89%) patients had detectable plasma viraemia during the study period. Time-course of plasma viraemia is shown in Fig. 1c. Only two patients had persistently negative plasma viraemia, both with detectable ICD-p24 Ag. Eight out of 13 (61.5%) patients with detectable plasma viraemia had  $>1 \log_{10}$  decrease in titre from baseline value during follow-up. A transient decrease in plasma viraemia was observed after 1 month of zidovudine therapy ( $P=0.10$ ) and lasted until month 7 of therapy before returning to pretreatment values (Fig. 1c).

Cellular viraemia was detected in all patients at baseline and during follow-up. Baseline TCID<sub>50</sub> mean titre was  $230 \pm 48.5$  per  $10^6$  cells and no marked trend could be identified during follow-up (Fig. 1d). Five out of 18 (28%) patients had  $>1 \log_{10}$  decrease in cellular viraemia titre from baseline value during the first 4 months of therapy, and were defined as responders. No correlation was found between ICD-p24 antigen levels and cellular or plasma viraemia titres at baseline and during follow-up (data not shown).

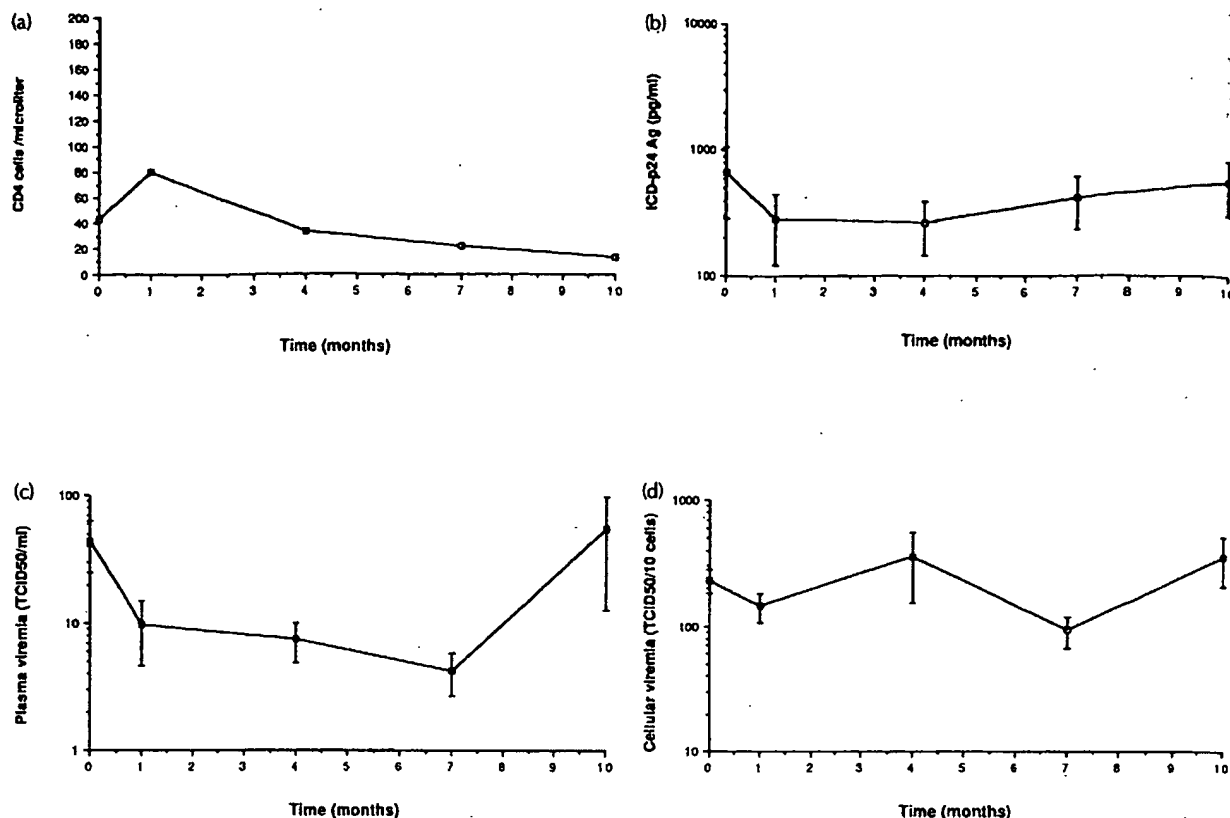


Fig. 1. (a) Cross-sectional medians of the CD4 cell counts; (b) mean immune complex-dissociated HIV-1 p24 antigen levels; (c) mean plasma and (d) cellular viraemia titres in 18 patients receiving zidovudine at day 0. Data are provided as mean  $\pm$  SE. TCID<sub>50</sub>, median tissue-culture infective dose.

#### Association between laboratory markers and clinical progression

Thirteen patients (72%) showed clinical progression during follow-up. Ten patients developed new AIDS-defining events: cerebral toxoplasmosis in three, disseminated cytomegalovirus infection in two, and PCP, tuberculous meningitis, cryptococcal meningitis, disseminated *Mycobacterium avium* infection and severe vacuolar myelopathy in one patient each. Five of these 10 patients died during follow-up. Three other deaths were recorded, but were not directly related to HIV in two patients (one committed suicide and one died of cirrhosis due to chronic hepatitis C virus infection); the third patient died of unknown cause. The cumulative incidence of progression to an AIDS-defining event was determined according to the Kaplan-Meier method (Fig. 2). The median time for progression to a new AIDS-defining event was 14 months in our study. Prognostic factors at baseline for development of a new AIDS-defining event included ICD-p24 Ag ( $P=0.04$ ), CD4 ( $P=0.0002$ ) and CD8 cell counts ( $P=0.004$ ). On multivariate analysis, however, only CD4 cell count remained predictive ( $P=0.003$ ). None of these markers was predictive for survival. Finally, each laboratory marker was separately introduced in a Cox regression model as a time-depen-

% free of AIDS-related event

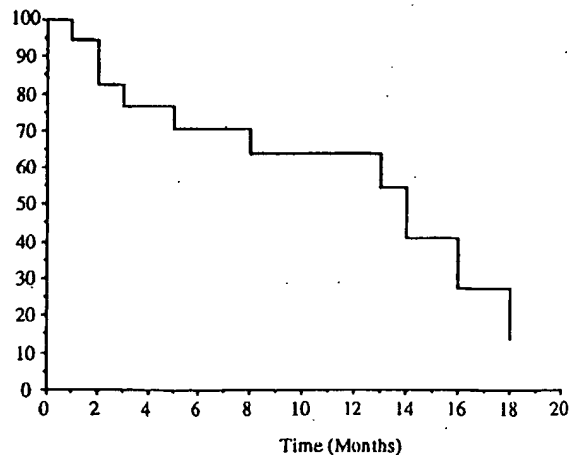


Fig. 2. Kaplan-Meier plot of the cumulative progression to an AIDS-defining event in 18 zidovudine-treated patients.

dent covariate to further examine the relationship between its value over time and clinical progression. The risk of developing a new AIDS-defining event appeared to be modified according to the current value of CD4 ( $P=0.002$ ) and CD8 cell counts ( $P=0.001$ ). However, neither was able to predict survival. Virologic response in ICD-p24 Ag or



plasma viraemia was not otherwise correlated with clinical outcome. Cellular viraemia responders, however, had a 5.6-fold decrease in their relative risk of developing a new AIDS-defining event (90% confidence interval, 1–33;  $P=0.05$ ).

## Discussion

We prospectively followed a cohort of 18 patients with symptomatic HIV infection and CD4 cell counts  $<350 \times 10^6/l$  to assess the prevalence of different viral markers, their changes under zidovudine therapy, and their relationship to clinical progression.

Plasma viraemia was detected in 13 of the 18 (72%) patients at baseline, and three additional patients had detectable plasma viraemia during follow-up. This high prevalence of plasma viraemia (89%) is not surprising since these patients had a median CD4 cell count of only  $43 \times 10^6/l$  at baseline. The sensitivity of plasma viraemia detection is correlated with CD4 cell counts [5,6,13]. Plasma viraemia has been found to be positive in  $>85\%$  of patients with CD4 cell counts  $<200 \times 10^6/l$ , and the overall rate of positive cultures in this study is similar to that reported in patients with AIDS [5,6,13]. The effect of antiviral therapy on plasma viraemia has been not fully investigated. Only a limited number of studies with short-term follow-up have reported a reduction in plasma viraemia titres in patients receiving zidovudine [5,8,9,10,16,17], a combination of zidovudine and interferon [18] or dideoxyinosine [11]. We also observed a decrease in plasma viraemia titres that lasted for 7 months under therapy and then returned to pretreatment values. However, this decrease was not statistically significant and only eight out of 13 (61.5%) patients had more than one  $\log_{10}$  decrease in their plasma viraemia titre under zidovudine compared with baseline.

HIV can also be isolated from PBMC in up to 90% of HIV-infected patients [5,6]. Quantification of cellular viraemia demonstrated a correlation with clinical status. A pilot study of short-term evaluation of plasma and cellular viraemia titres in 13 HIV-infected individuals demonstrated a decrease, although cellular viraemia titres decreased less than those of plasma viraemia [17]. All patients in our study had detectable cellular viraemia at baseline and during follow-up. No statistically significant changes in cellular viraemia titres under zidovudine therapy were detected during follow-up. Five patients (28%), however, experienced more than one  $\log_{10}$  decrease in cellular viraemia titre under zidovudine.

ICD-p24 Ag was the third virological marker analysed in this study. HIV-1 p24 Ag was the first marker of viral replication used in clinical trials, and significant decreases in p24 antigen levels have been

observed under zidovudine therapy [19,20]. Unfortunately, p24 antigen is not an ideal marker since it is only detected in 10–15% of asymptomatic HIV-infected patients, and 30–50% of symptomatics. Furthermore, its prognostic value appeared weaker in untreated patients than other markers such as CD4 cell counts [7]. A correlation between decrease of p24 antigen and clinical progression has yet to be identified and a direct relationship between p24 antigen and production of infectious virus is also unclear. Recent progress has been made in measuring p24 antigen associated with p24 antibodies in immune complexes, using the ICD-p24 Ag measure [21]. This new technique will increase the sensitivity of p24 antigen detection, thus increasing its potential interest as a surrogate marker. Thirteen of the 18 (72%) patients in our study had detectable ICD-p24 Ag, which was similar to the prevalence of plasma viraemia and twice as sensitive as the regular p24 antigen assay (data not shown). A decrease in ICD-p24 Ag was observed during the first 7 months of zidovudine therapy, but was not statistically significant. Eight patients (61.5%) had a virological response, defined as  $>50\%$  decrease of baseline value, after 1 month of zidovudine. Therefore, it appears that ICD-p24 Ag and plasma viraemia were detected in the same number of patients and followed similar patterns under zidovudine.

When these virological parameters were tested as baseline prognostic markers for disease progression, only ICD-p24 Ag correlated with clinical outcome in a univariate model, although this prognostic value disappeared in a multivariate analysis. Similarly, using time-dependent covariates, neither plasma viraemia nor ICD-p24 Ag titres correlated with clinical outcome. In this study, more than 60% of such patients were virological responders to zidovudine therapy as assessed by quantitative plasma cultures and ICD-p24 Ag. However, no clear relationship between virological response and clinical events such as the occurrence of a new AIDS-defining event was identified.

There are several possible reasons for these results. First, the sample size of the study may have been too small to detect a slight difference in new AIDS-defining events or survival. Alternatively, the severe immune deficiency observed in the patients with advanced HIV infection (median CD4,  $43 \times 10^6/l$ ; 94% having a previous diagnosis of AIDS), may have been only transiently affected by antiviral treatment, so that clinical progression could not be prevented despite an antiviral effect. The reason why some patients failed to show a virological response to zidovudine is also unclear. Zidovudine-resistant strains could have emerged relatively rapidly in our severely immunosuppressed patients after starting treatment. However, all patients were zidovudine-naïve at baseline and zidovudine resistance could only partly explain the lack of early virological re-

sponse in some patients. Differences in viral burden or in viral phenotype, however, might have played an important role. Recent data suggest that patients with syncytia-inducing strains do not respond to antiviral therapy [22].

Only patients with more than one log<sub>10</sub> decrease in cellular viraemia titres (28% of our population) appeared to have a statistically significant decrease in their risk of developing a new AIDS-defining event. If reduction of cellular viraemia titres under zidovudine is confirmed and is correlated with disease progression, it could be an interesting surrogate end-point that could be used in almost all HIV-infected patients. Plasma viraemia, on the other hand, does not appear to be of future interest, since in our study it was no more sensitive than ICD-p24 Ag, nor more predictive of disease progression. It will be interesting to see if other quantitative viral markers, such as the quantitation of proviral DNA and plasma viral RNA by polymerase chain reaction, would better correlate with disease progression.

The best predictors of clinical outcome in our study remained the immunological parameters. Indeed, CD4 cell count was the best baseline and time-dependent prognostic marker of disease progression. CD8 cell count also appears to be an interesting time-dependent predictor for development of a new AIDS-defining event in this population, although CD8 cell counts were not statistically affected by zidovudine therapy. CD8 lymphocytes have been reported to inhibit HIV replication *in vitro*, and decreased CD8 cell antiviral activity has been associated with disease progression [23]. CD8 cell counts, however, have not been identified as a prognostic marker for disease progression in patients with asymptomatic disease, regardless of whether they were receiving zidovudine [3,7,24]. The situation might be different in patients with advanced HIV infection, in whom immunological deterioration is likely to play a greater role than viral burden with regard to disease progression. These CD8 cells could also have broad antiviral efficacy against other viral infections like cytomegalovirus.

In conclusion, our findings suggest that immunological markers would be stronger or at least earlier predictors of clinical progression than virological markers in patients with symptomatic HIV infection treated with zidovudine. Only the decrease in cellular viraemia appeared to be associated with a decreased relative risk of subsequent development of a new AIDS-defining event. Results from ongoing studies are needed to validate these results, and the possibility of using changes in HIV cellular viraemia titres as a marker for the efficacy of antiviral therapy should be further investigated.

## Acknowledgements

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Bib Data Sheet

CONFIRMATION NO. 6514

<b>SERIAL NUMBER</b> 09/893,604	<b>FILING OR 371(c) DATE</b> 06/29/2001 <b>RULE</b>	<b>CLASS</b> 435	<b>GROUP ART UNIT</b> 1648	<b>ATTORNEY DOCKET NO.</b> BIOT1-11
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**APPLICANTS**

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**\*\* CONTINUING DATA \*\*\*\*\***

This appln claims benefit of 60/215,075 06/30/2000

**\*\* FOREIGN APPLICATIONS \*\*\*\*\***

**IF REQUIRED, FOREIGN FILING LICENSE GRANTED \*\***  
08/16/2001

**\*\* SMALL ENTITY \*\***

Foreign Priority claimed <input type="checkbox"/> yes <input type="checkbox"/> no	<b>STATE OR COUNTRY</b> MD	<b>SHEETS DRAWING</b> 0	<b>TOTAL CLAIMS</b> 16	<b>INDEPENDENT CLAIMS</b> 2
35 USC 119 (a-d) conditions met <input type="checkbox"/> yes <input type="checkbox"/> no <input type="checkbox"/> Met after Allowance				
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Methods for characterizing the viral infectivity status of a host

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(FILE 'HOME' ENTERED AT 13:19:40 ON 23 JAN 2005)

FILE 'MEDLINE' ENTERED AT 13:19:54 ON 23 JAN 2005

L1 148638 S (HIV OR HUMAN IMMUNODEFICIENCY VIRUS)  
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L3 9248 S L1 AND PY=1995  
L4 1 S L2 AND PY=1995  
L5 2 S L2 AND PY=1997  
L6 4 S L2 AND CD4?  
L7 0 S L2 AND GP120?

FILE 'USPATFULL' ENTERED AT 14:38:11 ON 23 JAN 2005

E HALLOWITZ R A/IN  
L8 7 S E4 OR E5  
L9 23222 S (MAGNETIC PARTICLE? OR MAGNETIC BEAD? OR PARAMAGNETIC  
PARTICL  
L10 4439 S L9 AND (HIV OR HUMAN IMMUNODEFICIENCY VIRUS)  
L11 521 S L10 AND AY<1996  
L12 64 S L11 AND GP120  
L13 63 S L12 AND ANTIBOD?  
L14 53 S L13 AND CD4?  
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L16 48 S L14 NOT L15  
L17 6 S L16 AND GP120/CLM  
L18 7 S L16 AND CD4?/CLM  
L19 7 S L18 NOT L17

FILE 'MEDLINE' ENTERED AT 07:18:50 ON 24 JAN 2005

L1 148638 S (HIV OR HUMAN IMMUNODEFICIENCY VIRUS)  
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PARAMAGNETIC  
L3 24 S L2 AND PY<1997  
L4 1 S L3 AND GP120  
L5 23 S L3 NOT L4  
L6 1470 S L2 AND GP120 OR GP160  
L7 5 S L2 AND (GP120 OR GP160)  
L8 47 S L2 NOT (L7 OR L4)  
L9 1617 S L1 AND (QUANTIF?)  
L10 52 S L9 AND GP120  
L11 42 S L10 AND PY<2002  
E HALLOWITZ R/AU  
L12 4 S E3 OR E4  
E KROWKA J/AU  
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E MATLOCK S/AU  
L15 3 S E4 OR E5  
L16 3 S L15 NOT L13  
L17 0 S L1 AND (INFECTIVITY STATUS)  
L18 8 S INFECTIVITY STATUS  
L19 4 S L1 AND (CELL-SURFACE GP120)  
L20 2692 S L1 AND (CD4? AND GP120)  
L21 0 S L20 AND (MARKERS OF DISEASE PROGRESSION)  
L22 102 S L20 AND (DISEASE PROGRESSION)

Serial No.: 09/893,604  
Applicants: Hallowitz, J. K., et al.

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L23      82 S L22 AND PY<2002
L24     1433 S L1 AND GP120/TI
L25      220 S L24 AND EXPRESSION
L26      26 S L25 AND DETECT?
L27       3 S L24 AND (ANTIGEN CAPTURE)

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L28      23222 S (MAGNETIC PARTICLE? OR MAGNETIC BEAD? OR PARAMAGNETIC
PARTICL
L29      4439 S L28 AND (HIV OR HUMAN IMMUNODEFICIENCY VIRUS)
L30      666 S L29 AND GP120
L31      51 S L30 AND GP120/CLM
L32      12 S L31 AND (MAGNETIC/CLM OR PARAMAGNETIC/CLM)
L33      37 S L30 AND (MAGNETIC/CLM OR PARAMAGNETIC/CLM)
L34      25      S      L33      NOT      L32

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L11 ANSWER 38 OF 42 MEDLINE on STN

91090988. PubMed ID: 2265028. Interactive laser cytometric analysis of retroviral protein expression in HIV-infected lymphocytic cell lines. Warren J T; McMahon J B; Weislow O S; Gulakowski R J; Kiser R F; Boyd M R. (Program Development Research Group (PDRG), National Cancer Institute, Frederick Cancer Research and Development Center (NCI-FCRDC), MD 21701. ) AIDS research and human retroviruses, (1990 Sep) 6 (9) 1131-7. Journal code: 8709376. ISSN: 0889-2229. Pub. country: United States. Language: English.

AB We have used interactive laser cytometry to investigate the expression of human immunodeficiency virus (HIV) envelope glycoproteins gp160, gp41, gp120, and the core protein p24 in the HIV-infected human lymphocyte cell lines H-9, CEM-SS, and C8166. This method allowed for the ultrasensitive detection of fluorescence signals at the single cell level and, when combined with specific anti-HIV antibodies, permitted unique quantitative detection of HIV antigens. Indirect immunofluorescence assays with monoclonal antibodies directed against gp120 revealed that a large proportion of lymphocytic cells expressed increased gp120-associated fluorescence consistent with HTLV-III<sub>RF</sub> infection. Certain monoclonal and polyclonal antibodies were also effective in quantifying gp160, gp41, and p24 expression. Expression of these antigens was found to vary significantly within 48 h. Significant loss (greater than or equal to 50%) of gp120 expression was observed when cells were treated with 1.0 microM AZT. The expression of the HIV-associated protein markers gp160, gp41, and p24 was detectable 24 h after infection of C8166, a cord blood lymphocytic cell line. C8166 cells expressed an additional 6- to 10-fold increase in gp120 in 48 h as well as a 3- to 4-fold increase in gp160, gp41, and p24. AZT (0.01 and 0.1 microM) decreased the expression of gp120, gp160, and p24 in a dose-dependent fashion. This new application of interactive laser cytometry permits early, sensitive, and statistically based distinctions in the expression of HIV-associated antigens in infected target cells at the single-cell level, and allows detection of important changes in HIV-associated antigen expression and the kinetics thereof. (ABSTRACT TRUNCATED AT 250 WORDS)

L14 ANSWER 22 OF 26 MEDLINE on STN

89119724. PubMed ID: 3219786. Interrelations of lymphocyte subset values, human immunodeficiency virus antibodies, and HIV antigen levels of homosexual males in San Francisco. Krowka J F; Stites D P; Moss A R; Casavant C H; Carlson J R; Chermann J C; Barre-Sinoussi F; Rodgers R P; Bacchetti P; McHugh T M; +. (Department of Laboratory Medicine, University of California, San Francisco 94143. ) Diagnostic and clinical immunology, (1988) 5 (6) 381-7. Journal code: 8705862. ISSN: 0895-0458. Pub. country: United States. Language: English.

AB Serum and leukocytes from a cohort of homosexual males were analyzed to determine the interrelationships of antibodies to human immunodeficiency virus (HIV), serum HIV antigen levels, and phenotypical differences in lymphocyte subpopulations of HIV antibody-positive (HIV Ab+) and HIV antibody-negative (HIV Ab-) homosexual males. Significant reductions were observed in the percentages of B lymphocytes, CD4+ and CD4+ kappa lambda- T lymphocytes and the CD4+/CD8+ ratios of HIV Ab+ homosexual males in comparison to HIV Ab- homosexual males. Significant increases were observed in the percentages of CD8+, CD8+ CD11b-, CD8+ kappa lambda-, CD8+ DR+, CD8+Leu7+, and Leu7+ lymphocytes of HIV Ab+ study subjects. Statistical analysis revealed that among the immunological variables tested, decreases in the CD4+/CD8+ ratio and in the percentage of CD4+ kappa lambda- lymphocytes showed the strongest associations with HIV-sero-positivity in asymptomatic homosexual males. Only 44 (16.5%) of 267 HIV Ab+ homosexual males had detectable levels of HIV antigen (HIV Ag) in their serum. The percentages of CD4+ or CD4+ kappa lambda- lymphocytes and the CD4+/CD8+ ratios of HIV Ab+ males differed significantly between HIV Ag-positive

(HIV Ag+) and--negative (HIV Ag-) homosexual males. These variables, however, did not correlate well with HIV Ag levels, indicating that no clear associations can be drawn between levels of HIV antigen and lymphocyte subset abnormalities of HIV-infected individuals.

L23 ANSWER 81 OF 82 MEDLINE on STN

90142677. PubMed ID: 2575824. Autoantibodies against CD4- and CD8-positive T lymphocytes in HIV-infected hemophilia patients. Daniel V; Weimer R; Schimpf K; Opelz G. (Department of Transplantation Immunology, University of Heidelberg, FRG. ) Vox sanguinis, (1989) 57 (3) 172-6. Journal code: 0413606. ISSN: 0042-9007. Pub. country: Switzerland. Language: English.

AB The presence of IgG, IgM, C3d, or gp120 on the surface of T lymphocytes was analyzed by flow cytometry in blood samples from 73 hemophilia patients and 56 healthy controls. IgG and IgM autoantibodies against CD4+ lymphocytes were found in HIV + patients but not in HIV-patients or healthy controls (p less than 0.001). IgM autoantibodies were more frequent than IgG autoantibodies. Autoantibody formation increased with disease progression. However, within the same disease risk category, patients with autoantibodies were not "more immunologically abnormal" than patients without autoantibodies. HIV + patients who possessed autoantibodies had similar CD4+ and CD8+ lymphocyte counts as HIV + patients without autoantibodies. There was no significant difference in the number of patients with abnormal CD4/CD8 ratios, serum neopterin levels, or in vitro responses to allogeneic stimulator cells or mitogens between autoantibody-positive or -negative patients of the same risk category. Our data suggest that autoantibodies against CD4+ lymphocytes may be helpful as indicators of disease progression, however, their immunopathogenetic role remains unclear.

L23 ANSWER 66 OF 82 MEDLINE on STN

96087587. PubMed ID: 8537109. Sequential occurrence of IgM, IgM/IgG, and gp120-IgM/IgG complement complexes on CD4+ lymphocytes in relation to CD4+ blood lymphocyte depletion in HIV+ hemophilia patients: results of a 10-year study. Daniel V; Susal C; Weimer R; Zipperle S; Kropelin M; Zimmermann R; Huth-Kuhne A; Opelz G. (Department of Transplantation Immunology, Institute of Immunology, Heidelberg, Germany. ) Immunology letters, (1995 Jul-Aug) 47 (1-2) 97-102. Journal code: 7910006. ISSN: 0165-2478. Pub. country: Netherlands. Language: English.

AB The concept of autoimmune mechanisms playing an integral role in the pathogenesis of HIV disease is rapidly gaining ground. In this study, we determined IgM and IgG antibodies, complement fragments and gp120 on the surface of CD4+ lymphocytes using double-fluorescence flow cytometry. Sequential analysis demonstrated an inverse relationship of autoantibodies and CD4+ lymphocyte counts in the peripheral blood. HIV+ patients without autoantibodies (16/104 = 15%) had the highest CD4+ blood cell counts (324 +/- 264/microliters; mean +/- SD). CD4+ counts were successively lower in patients with complement-fixing IgM (243 +/- 240/microliter), complement-fixing IgG and IgM (139 +/- 138/microliter), or gp120-IgM/IgG complement complexes on the surface of CD4+ cells (38 +/- 45/microliter, P = 0.03). Individual patient profiles show that IgM autoantibodies typically are formed early after HIV infection and appear to deplete CD4+ lymphocytes very slowly, whereas complement-fixing IgG autoantibodies are generated at a later stage and deplete CD4+ lymphocytes more efficiently. The presence of both soluble gp120 and complement-fixing autoantibodies on CD4+ lymphocytes is associated with very low CD4+ cell counts and coincides with progression to terminal disease. Early during HIV infection autoantibody production is rather unstable, but it becomes more stable with disease progression and persists in advanced stages of the disease. These data suggest that autoantibody formation against CD4+ lymphocytes is a pathogenic mechanism for CD4+ cell depletion.



L23 ANSWER 62 OF 82 MEDLINE on STN

96196114. PubMed ID: 8627018. CD4 masking during human immunodeficiency virus type 1 infection, quantified on peripheral blood lymphocytes, is a potential marker of disease progression. Carriere D; Vendrell J; Berthier A; Atoui N; Salhi S L; Reynes J; Gros P; Fontaine C; Jansen A; Huguet M; Ait-Cheik L; Pau B. (Sanofi Recherche (Laboratoire d'Immunologie), Montpellier, France. ) Journal of infectious diseases, (1996 Mar) 173 (3) 565-73. Journal code: 0413675. ISSN: 0022-1899. Pub. country: United States. Language: English.
- AB In human immunodeficiency virus type 1 (HIV-1)-infected adults, the proportion of gp120-free CD4 molecules on the surface of T lymphocytes was measured by double-epitope EIA and expressed as a CD epitope concentration ratio. In 51% of these patients (n=81), CD4 T cells showed a significant decrease (up to 100%) in the accessibility of the CD4 epitope in the D1 domain remained accessible. Of interest, a significant increase in the CD4 gp120 binding site concentration, without a change in T cell counts, was observed within 10 days after initiation of zidovudine treatment. Furthermore, CD4 masking by gp120 was associated with a poor clinical patient status. The assessment of the CD4 epitope concentration ratio is proposed as a surrogate marker of disease progression in HIV-1-infected patients.

L23 ANSWER 59 OF 82 MEDLINE on STN

96302269. PubMed ID: 8673544. Early clinical markers and CD4 percentage in subjects with human immunodeficiency virus infection. Wallace M R; Moss R B; Beecham H J 3rd; Grace C J; Hersh E M; Peterson E; Murphy R; Shepp D H; Siegal F P; Turner J L; Safrin S; Carlo D J; Levine A M. (U.S. Naval Medical Center-San Diego, California, USA. ) Journal of acquired immune deficiency syndromes and human retrovirology : official publication of the International Retrovirology Association, (1996 Aug 1) 12 (4) 358-62. Journal code: 9501482. ISSN: 1077-9450. Pub. country: United States. Language: English.
- AB In a clinical trial involving asymptomatic, HIV-seropositive subjects treated with either the HIV-1 immunogen (an inactivated, gp120-depleted HIV-1 virus in incomplete Freund's adjuvant) or an adjuvant control, we examined the relationship between changes in the percentage of CD4 cells over time and early clinical markers of HIV disease progression. Subjects who had an early clinical event were more likely to have a greater decline in the percentage of CD4 cells than those subjects who did not have a clinical event (p = 0.054). The greatest decline in CD4 percentage occurred within 10 weeks prior to a clinical event (mean 11% decrease from baseline). Subjects from the quartile with the greatest decline in CD4 percentage had a fivefold greater risk of having a clinical event than subjects from the quartile with the second largest decline (p = 0.045). These results demonstrate a relationship between changes in the percentage of CD4 cells and early clinical events. Further validation of this association may be useful in clinical monitoring and in evaluating therapies to treat HIV infection.

L26 ANSWER 25 OF 26 MEDLINE on STN

91376371. PubMed ID: 1896637. Trypsin-resistant gp120 receptors are upregulated on short-term cultured human epidermal Langerhans cells. Dezutter-Dambuyant C; Schmitt D A; Dusserre N; Hanau D; Kolbe H V; Kieny M P; Gazzolo L; Mace K; Pasquali J L; Olivier R. (INSERM U209, Laboratoire de Recherche Dermatologique et Immunologie, Hopital Edouard Herriot, Lyon, France. ) Research in virology, (1991 Mar-Jun) 142 (2-3) 129-38. Journal code: 8907469. ISSN: 0923-2516. Pub. country: France. Language: English.
- AB The CD4 molecule is known to be the preferential receptor for the HIV1 envelope glycoprotein. Epidermal Langerhans cells (LC) are dendritic

cells which express several surface antigens, among them the CD4 antigens. LC infection was suggested when these cells were seen to present buddings coincident with membrane thickening of roughly 100 nm in size. These buddings were similar in ultrastructural aspect to HIV buddings on in vitro infected promonocytic cells (U937). To clarify the exact role of CD4 molecules in LC infection induced by HIV1, we investigated the possible involvement of between native and recombinant HIV1 gp120 and the LC surface. We also assessed the expression of CD4 molecules on LC membranes dissociated by means of trypsin from their neighbouring keratinocytes. The cellular phenotype was monitored using flow cytometry. We show that human LC can bind the viral envelope protein and that this binding does not depend on CD4 protein expression. The amount of surface bound gp120 was not consistent with the amount of CD4 antigens present on LC membranes. The gp120-binding sites on LC in suspension appear to be trypsin-resistant while the CD4 antigens (at least the epitopes known to bind HIV1) are trypsin-sensitive. A burst of gp120 receptor expression was detected on 1-day cultured LC while the CD4 antigens disappeared. These findings lead to the logical conclusion that the binding of gp120 is due to the presence of a LC surface molecule which is different from CD4 antigens.

L5 ANSWER 22 OF 26 MEDLINE on STN

1999169169. PubMed ID: 10068574. Characterization of viral dynamics in human immunodeficiency virus type 1-infected patients treated with combination antiretroviral therapy: relationships to host factors, cellular restoration, and virologic end points. Wu H; Kuritzkes D R; McClermon D R; Kessler H; Connick E; Landay A; Spear G; Heath-Chiozzi M; Rousseau F; Fox L; Spritzler J; Leonard J M; Lederman M M. (Phase I Section, SDAC of ACTG, Frontier Science and Technology Research Foundation, Inc., Chestnut Hill, MA 02467, USA. wu@sdac.harvard.edu.) Journal of infectious diseases, (1999 Apr) 179 (4) 799-807. Journal code: 0413675. ISSN: 0022-1899. Pub. country: United States. Language: English.

AB Biphasic plasma viral decays were modeled in 48 patients treated with zidovudine, zalcitabine, and lamivudine. Estimated first- and second-phase decay rates were  $d_1$  as 0.47/day and  $d_2$  as 0.04/day. Interpatient differences in both decay rates were significant. The  $d_1$  was directly correlated with baseline CD4+, CD4+CD28+, and CD8+CD28+ T lymphocyte counts ( $P < .05$ ) and inversely correlated with baseline virus load ( $P = .044$ ) and the magnitude of CD4+ and CD8+ T lymphocyte recovery ( $P < .01$ ). The  $d_2$  was directly correlated with baseline percentage of CD8+ T lymphocytes ( $P = .023$ ), the CD8+CD38+ cell number ( $P = .024$ ), and the level of IgG that binds to human immunodeficiency virus (HIV) type 1 gp120 ( $P = .02$ ). Viral decay rates were not predictive of treatment failure or durability of viral suppression. These exploratory findings are consistent with a model in which immunologic factors contribute to elimination of HIV-infected cells and suggest a dynamic interplay between regulation of HIV expression and lymphocyte activation and recovery.

L5 ANSWER 5 OF 26 MEDLINE on STN

1999433493. PubMed ID: 10505674. The cell death-inducing ability of glycoprotein 120 from different HIV strains correlates with their ability to induce CD4 lateral association with CD95 on CD4+ T cells. Bottarel F; Feito M J; Bragardo M; Bonisconi S; Buonfiglio D; DeFranco S; Malavasi F; Bensi T; Ramenghi U; Dianzani U. (Department of Medical Sciences, A. Avogadro University of Eastern Piedmont at Novara, Italy.) AIDS research and human retroviruses, (1999 Sep 20) 15 (14) 1255-63. Journal code: 8709376. ISSN: 0889-2229. Pub. country: United States. Language: English.

AB CD4 cross-linking by HIV gp120 triggers CD4+ T cell death. Several authors have suggested that this effect is mediated by CD95, but this possibility is debated by other authors. In a previous work, we found by co-capping that gp120(451) and gp120MN, but not gp120(IIIB), induce lateral association of CD4 with CD95 on the T cell surface. In this work, we used fluorescence resonance energy transfer to confirm that CD4/CD95 lateral association is induced by gp120(451), but not gp120(IIIB). Moreover, we found that gp120 ability to induce the CD4/CD95 association correlates with ability to induce cell death, since gp120(451) and gp120MN induced higher levels of cell death than did gp120(IIIB) in PHA-derived CD4+ T cell lines. CD95 involvement in gp120-induced cell death was confirmed by showing that gp120(451) and gp120MN did not induce death in CD4+ T cells derived from patients with autoimmune/lymphoproliferative disease (ALD) and decreased CD95 function. Cell death induced by gp120MN was inhibited by a recombinant CD95/IgG.Fc molecule blocking the CD95/CD95L interaction. However, inhibition was late and only partial. These data suggest that the gp120-induced CD4/CD95 association exerts a dual effect: an early effect that is independent of CD95L and may be due to direct triggering of CD95 by gp120, and a late effect that may be due to sensitization of CD95 to triggering by CD95L. In line with the former effect, cell treatment with

gp120MN activated caspase 3 in the presence of Fas/IgG.Fc, which shows that cell death induced by gp120MN independently of CD95L uses the same pathway as CD95.

L8 ANSWER 15 OF 16 MEDLINE on STN

89271461. PubMed ID: 2567142. Predictive markers for the acquired immunodeficiency syndrome (AIDS) in hemophiliacs: persistence of p24 antigen and low T4 cell count. Eyster M E; Ballard J O; Gail M H; Drummond J E; Goedert J J. (Pennsylvania State University School of Medicine, Hershey. ) Annals of internal medicine, (1989 Jun 15) 110 (12) 963-9. Journal code: 0372351. ISSN: 0003-4819. Pub. country: United States. Language: English.

AB STUDY OBJECTIVE: To investigate the predictive value of assays for human immunodeficiency virus (HIV) p24 antigen, p24 antibody, and gp120 antibody compared with T4 cell counts. DESIGN: Prospective cohort selected from persons who had HIV-antibody seroconversion. PATIENTS: Eighty-seven persons with hemophilia with an actuarial cumulative acquired immunodeficiency syndrome (AIDS) incidence of 26% (CI, 12% to 40%), 8 years after HIV-antibody seroconversion. INTERVENTION: None. MEASUREMENTS AND MAIN RESULTS: Human immunodeficiency virus p24 antigen was detected in 8 of 74 (11%) of the patients without AIDS and 7 of 13 (54%) of the patients with AIDS. The 2-year actuarial incidence of AIDS was 24% (CI, 0% to 48%) after detection of p24 antigen, 16% (CI, 0% to 34%) after loss of p24 antibody, 20% (CI, 0% to 45%) after loss of gp120 antibody, 31% (CI, 15% to 47%) after a T4 count of less than 200 cells/microL, and 67% (CI, 31% to 100%) after a T4 count of less than 200 cells/microL among those patients positive for p24 antigen. Very low numbers of T4 and T8 lymphocytes, presence of p24 antigen in serum, and absence of p24 antibody all had some predictive value. However, only p24 antigen (relative hazard 6.0, P = 0.008) and T4 counts (relative hazard 5.3, P = 0.002 with T4 count less than 200 cells/microL) independently predicted AIDS up to 12 months before diagnosis. CONCLUSIONS: Strong predictors of AIDS are p24 antigenemia or low T4 counts. Detection of p24 antigen is highly specific and complementary to the greater sensitivity of low T4 counts. These findings have important implications regarding prognosis, counseling, and the planning of clinical trials.

L8 ANSWER 5 OF 16 MEDLINE on STN

95036227. PubMed ID: 7524737. Expression of CD4 by human hematopoietic progenitors. Louache F; Debili N; Marandin A; Coulombel L; Vainchenker W. (INSERM U 362, Institut Gustave Roussy, Villejuif, France. ) Blood, (1994 Nov 15) 84 (10) 3344-55. Journal code: 7603509. ISSN: 0006-4971. Pub. country: United States. Language: English.

AB It has been recently reported that murine hematopoietic stem cells and progenitors express low levels of CD4. In this study, we have investigated by phenotypic and functional analysis whether the CD4 molecule was also present on human hematopoietic progenitors. Unfractionated marrow cells or immunomagnetic bead-purified CD34+ cells were analyzed by two-color fluorescence with an anti-CD4 and an anti-CD34 monoclonal antibody (MoAb). A large fraction (25% to 50%) of the CD34+ cells was weakly stained by anti-CD4 antibodies. Moreover, in further experiments analyzing the expression of CD4 in different subpopulations of CD34+ cells, we found that CD4 was predominantly expressed in phenotypically primitive cells (CD34+ CD38-/low CD71low Thy-1high, HLA-DR+/low). However, the presence of CD4 was not restricted to these primitive CD34+ cell subsets and was also detected in a smaller fraction of more mature CD34+ cells exhibiting differentiation markers. Among those, subsets with myelo-monocytic markers (CD13, CD33, CD14, and CD11b) have a higher CD4 expression than the erythroid or megakaryocytic subsets. In vitro functional analysis of the sorted CD34+ subsets in colony assays and long-term

culture-initiating cell (LTC-IC) assays confirmed that clonogenic progenitors (colony-forming unit-granulocyte-macrophage, burst-forming unit-erythroid, and colony-forming unit-megakaryocyte) and LTC-IC were present in the CD4low population. However, most clonogenic progenitors were recovered in the CD4- subset, whereas the CD4low fraction was greatly enriched in LTC-IC. In addition, CD4low LTC-IC generated larger numbers of primitive clonogenic progenitors than did CD4- LTC-IC. These observations suggest that, in the progenitor compartment, the CD4 molecule is predominantly expressed on very early cells. The CD4 molecule present on CD34+ cells appeared identical to the T-cell molecule because it was recognized by three MoAbs recognizing different epitopes of the molecule. Furthermore, this CD4 molecule is also functional because the CD34+ CD4low cells are able to bind the human immunodeficiency virus (HIV) gp120. This observation might be relevant to the understanding of the mechanisms of HIV-induced cytopenias.

L14 ANSWER 27 OF 38 MEDLINE on STN

92348905. PubMed ID: 1640108. Evaluation of a flow cytometric model for monitoring HIV antigen expression in vitro. Heynen C A; Holzer T J. (Abbott Laboratories, Department of Experimental Biology Research, North Chicago, IL 60064. ) Journal of immunological methods, (1992 Jul 31) 152 (1) 25-33. Journal code: 1305440. ISSN: 0022-1759. Pub. country: Netherlands. Language: English.

AB Using flow cytometry, monoclonal antibodies to the HIV proteins p24, gp41 and p17 were evaluated for their ability to detect HIV antigens associated with HIV-infected T cells. Mixtures containing varying ratios of HIV-infected and uninfected cells were subjected to analysis with these monoclonal antibodies. In most cases, the monoclonal antibodies identified the correct ratio of HIV-infected cells to uninfected cells in the mixtures tested. An HIV anti-p24 monoclonal antibody was selected for further studies. Flow cytometric analysis was performed on various populations of cells including uninfected, acutely infected and chronically infected cells. Based on cell population fluorescence intensity three distinct regions were identified. In the first region were cells having low level fluorescence that were considered negative for HIV antigens, a profile detected in uninfected cells, and in the majority of cells in the first days following acute HIV infection. In the second region were those cells exhibiting strong fluorescence such as chronically infected cells or acutely infected cells several days after infection. A third region was identified containing cells that were intermediate in fluorescence intensity. Cells exhibiting intermediate intensity fluorescence appeared to have low concentrations of HIV p24 antigen associated with them either through viral adsorption and uptake or through low level virus expression. These intermediate region cells appeared in the early stages following acute infection, and also when chronically infected cells and uninfected cells were permeabilized together, suggesting a 'leaching' of HIV proteins from highly infected cells to uninfected cells. This leaching type of phenomenon could present problems in determining gating parameters for positive cells since uninfected cells that have associated HIV antigens exhibit higher fluorescence intensity than uninfected cells.

L24 ANSWER 91 OF 91 MEDLINE on STN

87310413. PubMed ID: 3305786. Correlation of serum HIV antigen and antibody with clinical status in HIV-infected patients. Paul D A; Falk L A; Kessler H A; Chase R M; Blaauw B; Chudwin D S; Landay A L. Journal of medical virology, (1987 Aug) 22 (4) 357-63. Journal code: 7705876. ISSN: 0146-6615. Pub. country: United States. Language: English.

AB An enzyme immunoassay (EIA) has been developed which detects antigen(s) (Ag) of the human immunodeficiency virus (HIV) in the serum of patients with the acquired immunodeficiency syndrome (AIDS), AIDS-related complex (ARC), and patients at high risk for HIV infection. The test

has a sensitivity of approximately 50 pg/ml of HIV protein. The specificity of the assay was determined with various virus infected cell lines, normal human sera/plasma, and serum from patients not known to be at risk for HIV infection. No false-positive HIV-Ag results were seen. Sera from 69% of patients with AIDS were positive for HIV-Ag as were 46% of patients with ARC and 19% of asymptomatic, HIV-antibody-positive individuals. There were significant associations between the stage of HIV infection--ie, AIDS vs ARC vs asymptomatic--and the detection of HIV-Ag in serum (p less than 0.0001) and the lack of detection of antibody to HIV core Ag (p less than 0.0001). HIV-Ag was also found in the serum of two asymptomatic antibody-negative individuals who were at high risk for AIDS and who later developed HIV antibody. The presence of HIV-Ag in sera was confirmed by an inhibition procedure. Thus, HIV-Ag can be detected in the serum of infected individuals prior to antibody production and correlates with the clinical stage of HIV infection.

L24 ANSWER 90 OF 91 MEDLINE on STN

88047956. PubMed ID: 3314657. Status of current clinical tests for human immunodeficiency virus (HIV): applications and limitations. Houn H Y; Pappas A A; Walker E M Jr. (Department of Pathology, University of Arkansas for Medical Sciences, Little Rock. ) Annals of clinical and laboratory science, (1987 Sep-Oct) 17 (5) 279-85. Ref: 31. Journal code: 0410247. ISSN: 0091-7370. Pub. country: United States. Language: English.

AB Two laboratory tests are currently used to detect the human immunodeficiency virus (HIV) specific antibodies that are produced when an individual has been infected by the virus at some time. These include the enzyme-linked immunosorbent assay (ELISA) as the screening test and the Western blot (WB) as the confirmatory test. They are not yet optimally effective and have brought with them some problems, especially when used to screen low risk populations such as asymptomatic blood donors. Currently licensed ELISA tests used to detect HIV have sensitivities that range between 93 percent and 99 percent, and all have specificities greater than 99 percent. An important concern is that the positive predictive value for the ELISA screening test is low in spite of the fairly high sensitivity and high specificity values. This poor predictive value is due to the low prevalence of individuals in the general population who have been infected with HIV. Multiple causes of false positive ELISA and Western blot tests have been identified. They can be eliminated by utilizing reagent antigens which are produced by recombinant deoxyribonucleic acid (DNA). The false negative ELISA and Western blot tests can be reduced by tests designed to detect IgM antibodies to HIV.

L24 ANSWER 86 OF 91 MEDLINE on STN

89328790. PubMed ID: 2502616. Comparison of two serum HIV antigen assays for selection of asymptomatic antigenemic individuals into clinical trials. Jackson J B; Sannerud K J; Balfour H H Jr. (Department of Laboratory Medicine and Pathology, University of Minnesota Health Science Center, Minneapolis 55455. ) Journal of acquired immune deficiency syndromes, (1989) 2 (4) 394-7. Journal code: 8812597. ISSN: 0894-9255. Pub. country: United States. Language: English.

AB The detection and recruitment of HIV antigen-positive asymptomatic individuals for clinical trials is important. Two commercial enzyme-linked immunosorbent assays (ELISA) for the detection and quantitation of human immunodeficiency virus (HIV) antigens were evaluated for sensitivity by testing serum samples from 155 asymptomatic HIV Western blot positive individuals. The Abbott HIV antigen ELISA detected HIV antigen in the serum of 17 (11.0%) of 155 patients compared with 18 (11.6%) of 155 by the Coulter HIV antigen ELISA. In serial twofold dilution experiments, there was no significant difference in sensitivity between these two assays in the detection of

HIV serum antigen. However, both assays are limited in their ability to detect HIV antigen in most asymptomatic HIV-infected patients. This low detection rate should be taken into account in the design of clinical trials involving asymptomatic infected patients.

L24 ANSWER 84 OF 91 MEDLINE on STN

90028766. PubMed ID: 2804364. Detection of HIV-1-infected cells from patients using nonisotopic in situ hybridization. Singer R H; Byron K S; Lawrence J B; Sullivan J L. (Department of Cell Biology, University of Massachusetts Medical School, Worcester 01655. ) Blood, (1989 Nov 1) 74 (6) 2295-301. Journal code: 7603509. ISSN: 0006-4971. Pub. country: United States. Language: English.

AB We have demonstrated that a sensitive, nonisotopic in situ hybridization (ISH) assay can be used to detect HIV-infected cells from seropositive, asymptomatic individuals. Our assay is based on the detection of a biotininated HIV DNA probe hybridized to human immunodeficiency virus (HIV)-infected peripheral blood lymphocytes (PBL) using streptavidin and alkaline phosphatase to identify positive cells. This assay is rapid in that it can be performed within a day and is sensitive enough to unambiguously identify a rare, single, positive cell. Patient samples derived from HIV-seropositive hemophiliacs and HIV-seropositive infants were analyzed before and after coculture with normal PBL. The same samples were investigated using a Dupont P24 antigen-capture kit. It was found that ISH always detected the same positive samples as antigen capture, often in shorter times of coculture. In situ hybridization detected over half of our HIV-infected hemophilia patient population as virus positive, whereas the antigen capture assay detected less than one fourth as virus positive. In situ hybridization detected positive cells directly, without coculture, in 12 out of 35 (34%) hemophiliacs and in three out of eight (37%) infants. The speed, sensitivity, and confidence of ISH and nonisotopic detection indicates that it will be useful as a tool for clinical research and diagnosis.

L24 ANSWER 78 OF 91 MEDLINE on STN

91162754. PubMed ID: 1672165. Few infected CD4+ T cells but a high proportion of replication-competent provirus copies in asymptomatic human immunodeficiency virus type 1 infection. Brinchmann J E; Albert J; Vartdal F. (Institute of Transplantation Immunology, National Hospital, Oslo, Norway. ) Journal of virology, (1991 Apr) 65 (4) 2019-23. Journal code: 0113724. ISSN: 0022-538X. Pub. country: United States. Language: English.

AB The virus load in CD4+ T cells from six asymptomatic human immunodeficiency virus type 1 (HIV-1)-infected individuals was determined by limiting-dilution analysis with a sensitive virus isolation procedure and the polymerase chain reaction (PCR). Both methods allowed detection of one HIV-1-infected cell among 10(5) uninfected cells. The number of provirus-containing CD4+ T cells was found to be 1 per 4,000 to 150,000 (median, 1 per 29,000), as determined by virus isolation and 1 per 2,500 to 26,000 (median, 1 per 12,000), as determined by PCR. Infected cells contained an average of 1 to 2 provirus copies, and a high proportion of the provirus copies (1 in 1 to 1 in 6; median, 1 in 2) were replication competent. The results suggest that only a few CD4+ T cells are likely to be lost as a direct consequence of the presence of HIV-1 in infected cells in asymptomatic individuals and that additional mechanisms may contribute to the depletion of CD4+ T cells observed in vivo.

L24 ANSWER 73 OF 91 MEDLINE on STN

92194459. PubMed ID: 1548759. Detection of replication-competent and

pseudotyped human immunodeficiency virus with a sensitive cell line on the basis of activation of an integrated beta-galactosidase gene. Kimpton J; Emerman M. (Program in Molecular Medicine, Fred Hutchinson Cancer Research Center, Seattle, Washington 98104. ) Journal of virology, (1992 Apr) 66 (4) 2232-9. Journal code: 0113724. ISSN: 0022-538X. Pub. country: United States. Language: English.

- AB We have constructed a HeLa cell line that both expresses high levels of CD4 and contains a single integrated copy of a beta-galactosidase gene that is under the control of a truncated human immunodeficiency virus type 1 (HIV-1) long terminal repeat (LTR). This cell line, called CD4-LTR/beta-gal, can be used to determine quantitatively the titer of laboratory-adapted HIV strains, and the method used to do so is as sensitive as the determination of viral titers in a T-cell line by end point dilution. Using this cell line as a titer system, we calculated that HIV-1 stocks contain only one infectious particle per 3,500 to 12,000 virions. Virus derived from a molecular clone of a macrophagetropic provirus will not infect this cell line. We have also cocultivated peripheral blood lymphocyte cultures from HIV-infected individuals with the CD4-LTR/beta-gal indicator cells. In a majority of primary isolates (five of eight), including isolates from asymptomatic patients, rare virus-infected cells that can activate the beta-galactosidase gene are present.

L24 ANSWER 67 OF 91 MEDLINE on STN

93037708. PubMed ID: 1417160. HIV screening and confirmation: a simplified and less expensive testing algorithm. Nkengasong J; Van Kerckhoven I; Carpels G; Vercauteren G; Piot P; van der Groen G. (Department of Infection & Immunity, Institute of Tropical Medicine, Antwerpen, Belgium. ) Annales de la Societe belge de medecine tropicale, (1992 Jun) 72 (2) 129-39. Journal code: 7511864. ISSN: 0365-6527. Pub. country: Belgium. Language: English.

- AB In this study we investigated the performance of fourteen different assays capable of simultaneously detecting antibodies to HIV-1 and HIV-2, referred to as combined screening assays (CSAs), on a panel of 371 sera, with a prevalence of 51.5% and 1.3% for HIV-1 and HIV-2 antibodies respectively. The geographic distribution of the sera was as follows; Europe (121), Africa (203) and Latin America (47). These sera were collected from different clinical groups of patients; Asymptomatic (36), AIDS-Related Complex/AIDS patients (18), infected individuals with generalised lymphadenopathy (12), blood donors (149), and subjects with unknown clinical status (156). The Dupont Western blot (WB) kit for detection of HTLV-III antibodies and the Pasteur new Lav-Blot II kit were used for the confirmation of HIV-1 and HIV-2 infection respectively. Of the 14 tests studied, 9 were enzyme linked immunosorbent assays (ELISAs), and 5 were non-Elisa tests requiring visual reading. An alternative approach for HIV antibody testing was studied retrospectively, whereby sera positive in an initial CSA (A) were retested on a second CSA (B), that was different from the first. The use of WB was limited to sera that gave discrepant (A+B-) results in the two CSAs. A positive result in both CSAs was reported as anti-HIV positive. A negative result in the first CSA was reported anti-HIV negative. Sensitivity, specificity, cost, and the delta (delta) values (delta values of the ELISA assays) were taken into consideration when selecting suitable pairs of assays. All the ELISAs scored 100% sensitivity, but for the non-ELISAs, the sensitivity ranged from 96.0% to 100%. The specificity for the ELISAs and non-ELISAs varied from 87.4% to 100% and from 51.4% to 100% respectively. Delta (delta) values for the ELISAs ranged from 3.82 to 136.68 and from -1.15 to -3.08 for the anti-HIV positive and anti-HIV negative populations respectively. Of the 121 test combinations studied, 9 (7.4%) pairs yielded 100% sensitivity and specificity and 61 (50.4%) pairs of CSAs required further testing on WB. This implies 100% positive predictive value, at a cost that was on average 6 times less, and a testing time that was 5 times faster than the



conventional algorithm. We conclude that there are several combinations of pairs of CSAs that can be used in the alternative algorithm that can provide accurate results at a much lower cost than the conventional algorithm requiring confirmation by WB of all initially reactive CSA results. (ABSTRACT TRUNCATED AT 400 WORDS)

L26 ANSWER 3 OF 38 MEDLINE on STN

2000294742. PubMed ID: 10836753. Dissociation of immunologic and virologic responses to highly active antiretroviral therapy. Fessel W J; Krowka J F; Sheppard H W; Gesner M; Tongson S; Weinstein S; Ascher M; Kwok S; Christopherson C. (Kaiser Permanente Medical Center, San Francisco, California 94115, USA. ) Journal of acquired immune deficiency syndromes (1999), (2000 Apr 1) 23 (4) 314-20. Journal code: 100892005. ISSN: 1525-4135. Pub. country: United States. Language: English.

AB OBJECTIVE: Immunologic markers, levels of HIV DNA, and infectious HIV were compared in partial responders (PR) to HAART who had high plasma HIV RNA levels but stable or increasing levels of CD4+ peripheral blood mononuclear cells (PBMC), and patients with complete failure (CF) who had very low or decreasing levels of CD4+ PBMC and high plasma HIV RNA levels. DESIGN AND METHODS: CD4 and CD8 levels were monitored by flow cytometry. Beta2-microglobulin (beta2M) and neopterin levels were measured by quantitative enzyme immunoassays. Plasma and PBMC from 11 PR and 13 CF were analyzed for infectious HIV levels in limiting dilution cultures. Polymerase chain reaction (PCR) assays were used to quantify cellular HIV DNA and plasma HIV RNA. RESULTS: In comparison with CF, PR had little or no CD4+ cell loss, a substantial increase in CD8+ cells, significantly fewer positive plasma HIV cultures ( $p = .03$ ), lower frequencies of infectious HIV in total PBMC ( $p = .005$ ) and in CD4+ PBMC ( $p < .001$ ), and lower frequencies of HIV DNA in CD4+ PBMC ( $p = .007$ ). CONCLUSIONS: Lower levels of infectious HIV and a lower frequency of CD4+ PBMC that contain "productive" HIV DNA in PR as compared with CF may contribute to the stable or increasing CD4+ PBMC levels of the PR. However, HAART may also have effects on lymphocyte homeostasis independent of its antiviral activity.

L31 ANSWER 47 OF 51 MEDLINE on STN

90345763. PubMed ID: 1696539. Flow cytometric method to monitor the destruction of CD4+ cells following their fusion with HIV-infected cells. Schols D; Pauwels R; Desmyter J; De Clercq E. (Rega Institute for Medical Research, Katholieke Universiteit Leuven, Belgium. ) Cytometry : journal of the Society for Analytical Cytology, (1990) 11 (6) 736-43. Journal code: 8102328. ISSN: 0196-4763. Pub. country: United States. Language: English.

AB Syncytium formation between HUT-78 cells persistently infected with human immunodeficiency virus type 1 (HIV-1) and uninfected CD4-bearing MOLT-4 or CEM cells results in a rapid destruction of the MOLT-4 or CEM cells. This syncytium formation is due to the interaction between the gp120 glycoprotein expressed by the persistently HIV-1-infected HUT-78 cells and the CD4 receptor present on MOLT-4 or CEM cells. A flow cytometric method has been applied to separate the infected (HUT-78) from the uninfected (MOLT-4, CEM) cell populations. This method is based on a modified DNA staining protocol which clearly shows the differences in DNA content between HUT-78 cells, on the one hand, and MOLT-4 or CEM cells, on the other hand. Using this flow cytometric method we have demonstrated that those compounds (i.e., sulfated polysaccharides, aurointricarboxylic acid) that interact with gp120 (of the HIV-infected cells) or CD4 (of the uninfected cells) suppress syncytium formation and concomitant destruction of the CD4+ cells.

L33 ANSWER 2 OF 4 BIOSIS COPYRIGHT 2004 BIOLOGICAL ABSTRACTS INC. on STN

1992:267495 Document No.: PREV199242126445; BR42:126445. SENSITIVITY SPECIFICITY AND PREDICTIVE VALUE OF DIAGNOSTIC TESTS DEFINITIONS AND CLINICAL APPLICATIONS. STRONGIN W [Reprint author]. CENT DRUG EVAL RES, FOOD DRUG ADM, ROCKVILLE, MD, USA. (1992) pp. 211-219. LENNETTE, E. H. (ED.). LABORATORY DIAGNOSIS OF VIRAL INFECTIONS, SECOND EDITION. XIII+783P. MARCEL DEKKER, INC.: NEW YORK, NEW YORK, USA; BASEL, SWITZERLAND. ILLUS. ISBN: 0-8247-8585-1. Language: ENGLISH.

L26 ANSWER 5 OF 5 MEDLINE on STN

93056534. PubMed ID: 1431129. CD4 changes conformation upon ligand binding. Szabo G Jr; Pine P S; Weaver J L; Rao P E; Aszalos A. (Center for Drug Evaluation and Research, FDA, Washington, DC 20204. ) Journal of immunology (Baltimore, Md. : 1950), (1992 Dec 1) 149 (11) 3596-604. Journal code: 2985117R. ISSN: 0022-1767. Pub. country: United States. Language: English.

AB Aurintricarboxylic acid (ATA) has been shown to block the binding site for both HIV gp120 and mAb anti-Leu 3a on CD4. We have unexpectedly found that brief treatment with > or = 1 micrograms/ml ATA rapidly disengages another mAb, OKT4E, after it has been bound to CD4 on human PBL. OKT4E is specific for a discontinuous epitope overlapping the MHC class II-binding region in the N-terminal CD4 domain. Interestingly, among 10 other mAb tested, only anti-Leu 8, specific for a leukocyte homing receptor is also quickly released from the cells by ATA treatment. Disengagement of the OKT4E mAb is also seen on a CD4-positive cell line (HPB-ALL) and with recombinant soluble CD4 (sCD4) bound to immobilized OKT4E. In all of these cases, disengagement is prevented if OKT4E is cross-linked, or the Leu 3a site is blocked by the mAb, but not by gp120. Photobleaching fluorescence resonance energy transfer (pFRET) measurements suggest that OKT4E is released as an indirect consequence of ATA-evoked conformational changes of CD4. Similar changes were detected as a result of gp120 binding to PBL. These data raise the possibility of a novel type of immunomodulation: induced disengagement of a bound ligand from its Ag.

L26 ANSWER 3 OF 5 MEDLINE on STN

95276119. PubMed ID: 7538802. Cross-linking of CD4 in a TCR/CD3-juxtaposed inhibitory state: a pFRET study. Szabo G Jr; Weaver J L; Pine P S; Rao P E; Aszalos A. (Department of Biophysics, University Medical School of Debrecen, Hungary. ) Biophysical journal, (1995 Mar) 68 (3) 1170-6. Journal code: 0370626. ISSN: 0006-3495. Pub. country: United States. Language: English.

AB Instances when T cell activation via the T cell receptor/CD3 complex is suppressed by anti-CD4 Abs are generally attributed either to the topological separation of CD4-p56lck from CD3, or their improper apposition. Photobleaching fluorescence resonance energy transfer measurements permitted direct analysis of these alternatives on human peripheral blood lymphocytes. Distinction between changes of relative antigen densities or positioning was made possible by simultaneously recording donor and acceptor fluorescence in the energy transfer experiment performed on homogeneous populations of flow-sorted cells. We show here that CD4 stays in the molecular vicinity of CD3, while anti-CD3 stimulation is suppressed by anti-CD4 or cross-linked HIV gp120. Our data suggest that cross-linking of CD4 through particular epitopes is capable of inhibiting activation driven by Abs binding to specific sites on CD3 without major topological sequestration of the Ags, in such a way that additional positive signals will also be affected. Thus, these and other related cases of negative signaling via CD4 may be interpreted in terms of functional uncoupling rather than a wide physical separation of CD4 from the T cell receptor-CD3 complex.

L26 ANSWER 1 OF 5 MEDLINE on STN

1999433493. PubMed ID: 10505674. The cell death-inducing ability of glycoprotein 120 from different HIV strains correlates with their ability to induce CD4 lateral association with CD95 on CD4+ T cells. Bottarel F; Feito M J; Bragardo M; Bonissoni S; Buonfiglio D; DeFranco S; Malavasi F; Bensi T; Ramenghi U; Dianzani U. (Department of Medical Sciences, A. Avogadro University of Eastern Piedmont at Novara, Italy. ) AIDS research and human retroviruses, (1999 Sep 20) 15 (14) 1255-63. Journal code: 8709376. ISSN: 0889-2229. Pub. country: United States. Language: English.

AB CD4 cross-linking by HIV gp120 triggers CD4+ T cell death. Several authors have suggested that this effect is mediated by CD95, but this possibility is debated by other authors. In a previous work, we found by co-capping that gp120(451) and gp120MN, but not gp120(IIIB), induce lateral association of CD4 with CD95 on the T cell surface. In this work, we used fluorescence resonance energy transfer to confirm that CD4/CD95 lateral association is induced by gp120(451), but not gp120(IIIB). Moreover, we found that gp120 ability to induce the CD4/CD95 association correlates with ability to induce cell death, since gp120(451) and gp120MN induced higher levels of cell death than did gp120(IIIB) in PHA-derived CD4+ T cell lines. CD95 involvement in gp120-induced cell death was confirmed by showing that gp120(451) and gp120MN did not induce death in CD4+ T cells derived from patients with autoimmune/lymphoproliferative disease (ALD) and decreased CD95 function. Cell death induced by gp120MN was inhibited by a recombinant CD95/IgG.Fc molecule blocking the CD95/CD95L interaction. However, inhibition was late and only partial. These data suggest that the gp120-induced CD4/CD95 association exerts a dual effect: an early effect that is independent of CD95L and may be due to direct triggering of CD95 by gp120, and a late effect that may be due to sensitization of CD95 to triggering by CD95L. In line with the former effect, cell treatment with gp120MN activated caspase 3 in the presence of Fas/IgG.Fc, which shows that cell death induced by gp120MN independently of CD95L uses the same pathway as CD95.

L13 ANSWER 49 OF 56 MEDLINE on STN

91084577. PubMed ID: 1824617. Autoantibodies against CD4 cells are associated with CD4 helper defects in human immunodeficiency virus-infected patients. Weimer R; Daniel V; Zimmermann R; Schimpf K; Opelz G. (Department of Transplantation Immunology, University of Heidelberg, FRG. ) Blood, (1991 Jan 1) 77 (1) 133-40. Journal code: 7603509. ISSN: 0006-4971. Pub. country: United States. Language: English.

AB To investigate whether autoantibodies against CD4-positive lymphocytes might induce helper dysfunction, autoantibody formation and T-cell function was examined simultaneously in 61 hemophilia patients. Twenty patients were human immunodeficiency virus (HIV)-negative, 26 HIV-positive stage CDC II or III, and 15 were HIV-positive stage CDC IV. T lymphocytes, CD4-positive, or CD8-positive T subsets were cocultured with B lymphocytes and pokeweed mitogen (PWM) for 6 days and Ig-secreting cells were assessed in a reverse hemolytic plaque assay. The presence of IgM, IgG, C3d, or gp120 on the surface of T cells or T subsets was analyzed by flow cytometry. Autoantibodies against CD4-positive T cells were not detected in controls or HIV-negative patients, but were common in HIV-positive patients (20 of 41 patients). In patients with autoantibodies we found an increased incidence of CD4 helper defects (P less than .0001 in CDC II or III patients; P less than .02 in CDC IV patients). 12 of 13 patients with IgM autoantibodies and 4 of 4 with IgG autoantibodies showed CD4 helper defects. Complement fixation had no relevance. Autoantibody formation against CD4 cells was not due to increased in vivo B-cell stimulation (spontaneous plaque formation: 611 +/- 204 PFC/10(6) B cells in autoantibody-negative patients

v 650 +/- 202 PFC/10(6) B cells in autoantibody-positive patients; not significant). Thus, our results suggest that autoantibody formation is not caused by a general state of in vivo B-cell activation. Rather, the production of autoantibodies appears to coincide with defects in B-cell proliferation or differentiation, as shown by reduced mitogen-stimulated B-cell responses in CDC II and III patients (P less than .05). Autoantibodies against CD4 cells appear to be involved in the pathogenesis of CD4 helper defects of HIV-infected patients.

L13 ANSWER 38 OF 56 MEDLINE on STN

93214034. PubMed ID: 8461467. Complement and virus-specific antibody-dependent infection of normal B lymphocytes by human immunodeficiency virus type 1. Gras G; Richard Y; Roques P; Olivier R; Dormont D. (Laboratoire de Neuropathologie Experimentale et Neurovirologie, Commissariat a l'Energie Atomique, Fontenay aux Roses, France. ) Blood, (1993 Apr 1) 81 (7) 1808-18. Journal code: 7603509. ISSN: 0006-4971. Pub. country: United States. Language: English.

AB We tested the susceptibility of human purified, normal B lymphocytes to human immunodeficiency virus type 1 (HIV-1) infection, in the presence or absence of complement-sufficient serum and of virus-specific antibodies. Virus replication was detected when cells were infected in the presence of both complement and anti-HIV antibodies (C'-ADE conditions), by day 2 postinfection. Similar results were obtained when B lymphocytes were purified either from peripheral blood (three healthy donors) or from tonsils (four individuals with chronic tonsillitis). HIV infection was shown by polymerase chain reaction (PCR) detection of proviral sequences (gag and pol genes), by p24 antigen synthesis, and by cocultivation assay with MT2 cells. The higher p24 production was obtained when B cells were preactivated for 2 days by phorbol 12-myristate 13-acetate (PMA) before infection and then cultured in the presence of low-molecular weight B-cell growth factor (LMW-BCGF). Expression of virus envelope glycoprotein (gp) 120 could also be detected on a subpopulation of B cells (CD19+, CD22+) by flow cytometry. Blocking experiments with monoclonal antibodies (MoAbs) against CD4, CD21 (complement receptor 2 [CR2]), CD35 (CR1), CD19, and CD5 surface molecules indicated that infection of B cells involves CD4, CD21, and CD35 antigens. Indeed, blocking of CD4 receptor inhibited 10% of p24 production, and blocking of both CD21 and CD35 led to extinction of p24 signal. CR-dependent pathway is thus a major route for C'-ADE of HIV infection in normal B cells. Our results emphasize the importance of studying interactions between HIV and the complement system for better understanding infection mechanisms and the major dysfunctions of B cells in HIV-infected individuals.

L5 ANSWER 37 OF 56 MEDLINE on STN

94014991. PubMed ID: 8409932. Detection of unintegrated human immunodeficiency virus type 1 DNA in persistently infected CD8+ cells. Mercure L; Phaneuf D; Wainberg M A. (McGill University AIDS Centre, Chemin Cote-Ste-Catherine, Montreal, Canada. ) Journal of general virology, (1993 Oct) 74 ( Pt 10) 2077-83. Journal code: 0077340. ISSN: 0022-1317. Pub. country: ENGLAND: United Kingdom. Language: English.

AB The presence of unintegrated viral DNA has been reported in cells persistently infected by lentiviruses, including human immunodeficiency virus type 1 (HIV-1). We confirm that CD8+ cells can be productively and persistently infected by HIV-1 for up to 4 months, as determined by secretion of viral core antigen p24 into the extracellular medium and by indirect immunofluorescence. The expression of the external viral glycoprotein gp120 at the surface of these cells was demonstrated by two-colour flow cytometry. Progeny virions recovered from CD8+ cells were infectious in CD4+ T cells. Despite an

absence of significant cytopathology, these chronically infected CD8+ cells were shown to harbour unintegrated HIV-1 DNA, as detected by quantitative PCR. Both linear and circular forms of the extrachromosomal viral genome were present in infected CD8+ cells, as early as 3 weeks before a peak in viral replication. These findings provide evidence that the presence of unintegrated viral DNA during lentiviral infection may not always correlate with c.p.e.

L5 ANSWER 25 OF 56 MEDLINE on STN

95264474. PubMed ID: 7745728. Serotyping of primary human immunodeficiency virus type 1 isolates from diverse geographic locations by flow cytometry. Zolla-Pazner S; O'Leary J; Burda S; Gorny M K; Kim M; Mascola J; McCutchan F. (Veterans Affairs Medical Center, New York, New York 10010, USA. ) Journal of virology, (1995 Jun) 69 (6) 3807-15. Journal code: 0113724. ISSN: 0022-538X. Pub. country: United States. Language: English.

AB The immunologic relatedness of the various human immunodeficiency virus type 1 (HIV-1) clades was determined with 13 human anti-HIV-1 monoclonal antibodies (MAbs) to six immunogenic regions of the HIV-1 structural proteins. The immunoreactivity of the native, oligomeric viral envelope glycoproteins expressed on the surfaces of human peripheral blood mononuclear cells infected in vitro with primary isolates from clades A through E was determined by flow cytometry. Some epitopes in the immunodominant region of gp41 and the C terminus of gp120 appear to be HIV-1 group specific in that they are expressed on the surfaces of cells in cultures infected with the majority of viruses tested from clades A to E. Epitopes within the V3 region appear to be clade restricted. Surprisingly, one MAb to an epitope in the C terminus of gp120 was entirely clade B specific. Staining with anti-V2 and anti-CD4 binding domain (CD4bd) reagents was infrequently detected. Anti-CD4bd MAbs stained only CD4-negative T cells because the CD4bd of gp120 appeared to be complexed with membrane CD4. When present, the epitopes of V2 and the CD4bd appeared to be expressed on cells infected with various clades. Thus, the results suggest that MAbs to gp41, the C terminus, and the V3 loop of gp120 are most useful in serotyping primary isolates of HIV-1, providing group-specific, clade-restricted, and clade-specific reagents. The use of the immunofluorescent method with the reagents described herein distinguishes infection with clade B from that with all other HIV-1 clades. With additional MAbs, this technique will allow a broadly applicable, reproducible, and practical method for serotyping HIV-1.

L5 ANSWER 7 OF 56 MEDLINE on STN

1999329163. PubMed ID: 10400736. Both memory and CD45RA+/CD62L+ naive CD4(+) T cells are infected in human immunodeficiency virus type 1-infected individuals. Ostrowski M A; Chun T W; Justement S J; Motola I; Spinelli M A; Adelsberger J; Ehler L A; Mizell S B; Hallahan C W; Fauci A S. (Laboratory of Immunoregulation, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892, USA.. mostrowski@nih.gov) . Journal of virology, (1999 Aug) 73 (8) 6430-5. Journal code: 0113724. ISSN: 0022-538X. Pub. country: United States. Language: English.

AB Cellular activation is critical for the propagation of human immunodeficiency virus type 1 (HIV-1) infection. It has been suggested that truly naive CD4(+) T cells are resistant to productive HIV-1 infection because of their constitutive resting state. Memory and naive CD4(+) T-cell subsets from 11 HIV-1-infected individuals were isolated ex vivo by a combination of magnetic bead depletion and fluorescence-activated cell sorting techniques with stringent criteria of combined expression of CD45RA and CD62L to identify naive CD4(+) T-cell subsets. In all patients HIV-1 provirus could be detected within

naive CD45RA+/CD62L+ CD4(+) T cells; in addition, replication-competent HIV-1 was isolated from these cells upon CD4(+) T-cell stimulation in tissue cultures. Memory CD4(+) T cells had a median of fourfold more replication-competent virus and a median of sixfold more provirus than naive CD4(+) T cells. Overall, there was a median of 16-fold more integrated provirus identified in memory CD4(+) T cells than in naive CD4(+) T cells within a given patient. Interestingly, there was a trend toward equalization of viral loads in memory and naive CD4(+) T-cell subsets in those patients who harbored CXCR4-using (syncytium-inducing) viruses. Within any given patient, there was no selective usage of a particular coreceptor by virus isolated from memory versus naive CD4(+) T cells. Our findings suggest that naive CD4(+) T cells may be a significant viral reservoir for HIV, particularly in those patients harboring CXCR4-using viruses.

L5 ANSWER 2 OF 56 MEDLINE on STN

2000186241. PubMed ID: 10721463. Application of monoclonal antibodies to monitor the synthesis of a glycoprotein core of envelope glycoproteins of human immunodeficiency virus (HIV-1). Jagodzinski P P; Trzeciak W H. (Department of Physiological Chemistry, University of Medical Sciences, Poznan, Poland. ) Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie, (2000 Feb) 54 (1) 50-3. Journal code: 8213295. ISSN: 0753-3322. Pub. country: France. Language: English.

AB Using monoclonal antibodies 0.5 beta or G3-42, directed against V3 and C4 domains of glycoprotein 120 (gp120), we monitored the synthesis of oligomeric and monomeric forms of HIV-1 envelope glycoprotein 120 by flow cytometry or immunoprecipitation analysis in chronically infected MoIT-4 cells, cultured in the presence of tunicamycin. We observed that the inhibition of glycosylation by high concentrations of tunicamycin results in the reduction of an oligomeric gp120 on the surface of infected MoIT 4 cells as well as the decrease in the concentration of a monomeric form in the cytoplasm. Our studies revealed that the antibody 0.5 beta (exhibited higher sensitivity in the detection of gp120 than the antibody G3-42). We also observed that both antibodies did not recognise nonglycosylated precursor core envelope protein.

94014991. PubMed ID: 8409932. Detection of unintegrated human immunodeficiency virus type 1 DNA in persistently infected CD8+ cells. Mercure L; Phaneuf D; Wainberg M A. (McGill University AIDS Centre, Chemin Cote-Ste-Catherine, Montreal, Canada. ) Journal of general virology, (1993 Oct) 74 ( Pt 10) 2077-83. Journal code: 0077340. ISSN: 0022-1317. Pub. country: ENGLAND: United Kingdom. Language: English.

AB The presence of unintegrated viral DNA has been reported in cells persistently infected by lentiviruses, including human immunodeficiency virus type 1 (HIV-1). We confirm that CD8+ cells can be productively and persistently infected by HIV-1 for up to 4 months, as determined by secretion of viral core antigen p24 into the extracellular medium and by indirect immunofluorescence. The expression of the external viral glycoprotein gp120 at the surface of these cells was demonstrated by two-colour flow cytometry. Progeny virions recovered from CD8+ cells were

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infectious in CD4+ T cells. Despite an absence of significant cytopathology, these chronically infected CD8+ cells were shown to harbour unintegrated HIV-1 DNA, as detected by quantitative PCR. Both linear and circular forms of the extrachromosomal viral genome were present in infected CD8+ cells, as early as 3 weeks before a peak in viral replication. These findings provide evidence that the presence of unintegrated viral DNA during lentiviral infection may not always correlate with c.p.e.

# Correlation between kinetics of soluble CD4 interactions with HIV-1-Env-expressing cells and inhibition of syncytia formation: implications for mechanisms of cell fusion and therapy for AIDS

Dimiter S. Dimitrov, Kathleen Hillman\*, Jody Manischewitz\*,  
Robert Blumenthal and Hana Golding\*

**Objectives:** To study the kinetics of the interactions between soluble (s) CD4 and HIV-1-Env-expressing cells in relation to subsequent events leading to cell fusion and inhibition of syncytia formation.

**Design:** Vaccinia-HIV-1 (Env)-infected CD4<sup>+</sup> T-cells were used to study the kinetics of sCD4-gp120/41 interactions and syncytia formation (with CD4<sup>+</sup> T-cells) under identical conditions.

**Methods:** sCD4 association and dissociation rates for HIV-1-Env-expressing cells, and quantification of sCD4-induced gp120 shedding was determined by a quantitative flow cytometry assay. Syncytia inhibition was measured in the continuous presence of sCD4, or after washing of HIV-1-Env-expressing cells following pre-incubation with sCD4.

**Results:** The kinetics of syncytia inhibition correlated with sCD4 binding when sCD4 was maintained during the culture. When Env-expressing cells, which had been pre-incubated with sCD4, were washed to remove unbound sCD4, no syncytia formation inhibition was observed, even following sCD4-induced shedding of > 50% of surface gp120 molecules.

**Conclusions:** The lack of syncytia inhibition seen after removal of unbound sCD4, even after pre-incubation of cells under saturation and gp120 shedding conditions, indicated that sufficient numbers of fusogenic molecules remained on the sCD4-treated cells. In addition, fast dissociation of pre-bound sCD4 occurred in culture. These results are important for understanding HIV-1-Env-mediated cell fusion and AIDS therapy.

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**Keywords:** sCD4 binding, gp120 shedding, syncytia inhibition, sCD4 treatment of AIDS, membrane fusion.

[For editorial comment, see pp. 345-347]

## Introduction

The soluble form of the HIV-1 receptor CD4 (sCD4), has been found to be a very potent inhibitor of *in vitro* viral infection and syncytia formation [1-6]. Recently, it has also been demonstrated that high concentrations of sCD4 (> 5 µg/ml) when incubated with intact virions or with infected cells causes slow gp120 shedding from the surface of virions or cells [7-12].

It has been proposed that this mechanism may lead to irreversible viral neutralization *in vivo*, which remains effective even after decline of serum sCD4 concentrations [7-12]. The sCD4 molecule or a chimeric CD4-immunoglobulin (Ig) Fc variant could therefore provide a powerful therapeutic *in vivo* agent which could block the spread of HIV-1 in infected individuals. Initial clinical trials have been disappointing, however, since no significant changes in surrogate markers

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or viral spread were observed [13–15]. This may have been due to reduced susceptibility of some primary HIV-1 isolates to sCD4, as has been demonstrated *in vitro* [16]. An alternative explanation relates to the rapid clearance of sCD4, which reaches concentrations of 50–300 ng/ml, and then declines within several hours after administration. Thus, the affinity of sCD4 for HIV-1-Env is not sufficient to ensure *in vivo* blocking of viral spread. Rather, the kinetics of sCD4 binding at different serum concentrations might be a more critical factor for the determination of the outcome of such treatment.

We therefore developed a sensitive assay which allowed us to monitor the kinetics of sCD4 binding and dissociation from cells as a function of sCD4 concentration [17]. The kinetics were compared with the kinetics of syncytia formation inhibition. We found that: (1) the kinetics of syncytia inhibition of sCD4 correlates well with the kinetics of binding at different sCD4 concentrations; (2) washing of Env-expressing cells after pre-incubation with sCD4 (prior to their coculture with CD4+ T-cells), results in no inhibition of syncytia formation, even at high sCD4 concentrations (2–20 µg/ml), which induces shedding of up to 70% of the surface gp120 molecules; and (3) the lack of inhibition is due to a rapid dissociation of bound sCD4 under these conditions, which is faster than the rate of cell fusion and syncytia formation.

## Material and methods

### Cell lines and reagents

The human cell line CEM was obtained from the American Type Culture Collection (ATCC; Rockville, Maryland, USA). The CD4<sup>+</sup> subclone 12E1 was derived from CEM cells by chemical mutagenesis with EMS followed by negative selection with the monoclonal antibody OKT4A plus complement [18]. The recombinant vaccinia virus vPE16 which expresses HIV-1<sub>III</sub>B-Env (gp120/41), was donated by P. Earl and B. Moss [National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH), Bethesda, Maryland, USA]. Fluorescein isothiocyanate (FITC)-conjugated OKT4 monoclonal antibody was obtained from Ortho Diagnostics (Raritan, New Jersey, USA). sCD4 [produced in Chinese hamster ovary (CHO) cells], and polyclonal rabbit anti-gp120 serum were obtained from American BioTechnology (ABT; Cambridge, Massachusetts, USA). FITC-goat anti-rabbit was obtained from Sigma (St Louis, Missouri, USA).

### Determination of gp120 expression and sCD4 binding to cells by flow cytometry

This assay has been described in detail previously [17]. Briefly, CD4<sup>+</sup> 12E1 cells were infected with recombinant vaccinia virus vPE16 at 10 p.f.u./cell (as determined in a HeLa cell plaque assay). Twenty hours later, cells were stained with rabbit anti-gp120 serum

followed by FITC-goat anti-rabbit, or were incubated with different sCD4 concentrations for 10–360 min at 37°C in a total volume of 100 µl. At different intervals, cells were washed and stained with 1 µg/ml FITC-OKT4 at 4°C. CD4<sup>+</sup> CEM cells were stained with FITC-OKT4 in parallel. In order to determine sCD4 dissociation, cells were incubated with sCD4 under saturation conditions, then washed three times to remove unbound sCD4, resuspended in 1 ml RPMI medium [supplemented with 10% fetal calf serum (FCS)], and incubated at 37°C with intermittent shaking. Cell aliquots were removed at different intervals and stained with FITC-OKT4. Stained cells were analyzed by flow cytometry using a fluorescent cell analyzer (Epic Profile; Coulter Counter, Hialeah, Florida, USA). The relative fluorescence intensities in fluorescent units (FU), were calculated as described previously [17]. This assay allowed us to estimate the numbers of gp120 molecules expressed on vPE16-infected 12E1 cells, and to follow the kinetics of sCD4 association and dissociation from HIV-1-Env-expressing cells after washing to remove any excess unbound sCD4, and continuous incubation at 37°C. It was determined in a preliminary study that surface expression of gp120/41 was stable between 20–24 h following vPE16-infection of 12E1 cells. There was no evidence for spontaneous shedding or marked expression of newly synthesized molecules, as determined by staining with rabbit anti-gp120 and rabbit anti-V3 polyclonal antibodies (ABT, data not shown).

### Inhibition of syncytia formation by soluble sCD4

vPE16-infected 12E1 effectors were incubated with sCD4 at different concentrations and for different time intervals at 37°C, as described above. At various intervals, these effectors were either washed three times to remove unbound sCD4, or were left unwashed, and mixed with CD4<sup>+</sup> CEM cells (targets) at a 1:1 ratio in 96-well plates. The unwashed effectors were cocultured with CEM cells in the continuous presence of sCD4 at the same concentration as during the pre-incubation stage. In some experiments, effectors were not pre-incubated with sCD4, but were mixed directly with CEM targets, and sCD4 added at time 0 or at different time points after initiation of coculture. Syncytia formation was microscopically monitored over a period of 4–8 h. Syncytia were defined as giant cells greater than four times the diameter of uninfected single cells [19].

In addition to the kinetics of syncytia formation, we also followed the kinetics of fusion between effector and target cells. For this purpose, vPE16-infected 12E1 cells were labeled with the lipid fluorophore DiI (3.5 mg/ml; Molecular Probes, Eugene, Oregon, USA) in order to monitor membrane mixing, or with the fluorescent aqueous dye BCECF (Molecular Probes) to monitor cytoplasmic continuity [20–22]. Cells were then washed three times and resuspended in complete RPMI medium supplemented with 10% FCS. These la-

beled effectors were mixed with CD4+ CEM cells at a 1:100 ratio and the cocultures were monitored undisturbed by fluorescence microscopy as described previously [20]. Fusion was defined as dye transfer between labeled effectors and adjacent target cells at different intervals. Dye transfer at time 0, or between effectors and CD4- cells, did not exceed 5–7% per culture.

#### Analysis of sCD4 dissociation kinetics

Dissociation was analyzed using an exponential function:

$$\text{sCD4} - R/\text{sCD4} - R_t = A \exp(-t/t_d) + B$$

$$t_d = 1/k_d$$

where sCD4 - R is the surface bound sCD4 ( $R = \text{gp120/41 Env molecules}$ ) and sCD4 -  $R_t$ , its value at time 0. A and B are constants which represent the dissociable and undissociable components of bound sCD4, respectively, and are independent of time  $t$ . ( $A + B = 1$ ).  $t_d$  is the characteristic time of dissociation, and  $k_d$  the corresponding dissociation rate constant.

Our experimental data were fitted to the kinetic equation using the Sigmaplot 4.0 computer program (Jandel Scientific, Corte Madera, California, USA). The constants were calculated from the best fits with correlation coefficients  $> 0.96$ . The maximal sCD4 surface concentration ( $\text{sCD4} - R_t = A + B$ ), was calculated from the surface sCD4 saturation concentration ( $\text{sCD4} - R$ ) which was determined experimentally (pre-incubation with 20  $\mu\text{g/ml}$  sCD4 for 10–15 min at 37°C, or for 2 h at 4°C).

## Results

#### Correlation between sCD4 binding to HIV-1-Env-expressing cells and syncytia inhibition

In order to assess the ability of sCD4 to block *in vivo* HIV-1 infection by cell-free virions or by cell-associated virus, several parameters must be considered.

- (1) The pharmacokinetics of sCD4 in the blood stream, that is the time required to achieve peak levels, and the rate of sCD4 clearance.
- (2) Kinetics of sCD4 binding to free virions and/or Env-expressing infected cells.
- (3) The kinetics of viral genome transfer by either virion to cell, or by cell-to-cell fusion.

To follow the kinetics of sCD4 interactions with HIV-1-infected cells, we developed a sensitive assay to follow the binding of sCD4 to HIV-1-Env-expressing cells. In this assay, the CD4- 12E1 subclone of the CEM line was infected with a HIV-1<sub>IIIIB</sub>-Env (gp120/41)-expressing recombinant vaccinia (vPE16). Twenty hours later, the cells were incubated with different sCD4 concentrations, and for different inter-

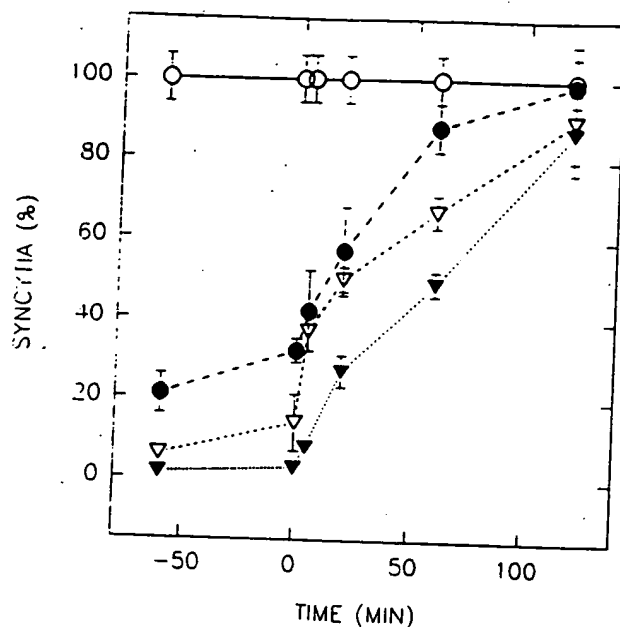
vals. In order to determine bound CD4, these cells were then analyzed by flow cytometry following staining with FITC-OKT4 (which binds to a CD4 site distal to the gp120 binding site) [17]. Treated cells were mixed with uninfected CD4+ CEM cells in parallel in order to follow syncytia formation.

Surface expression of gp120/41 on vPE16-infected cells was stable over an 8 h period in the absence of sCD4, as determined by staining with rabbit anti-gp120 polyclonal serum [17]. This binding assay allowed us to calculate both the affinity and the kinetics of sCD4 binding to Env-expressing cells under different conditions.

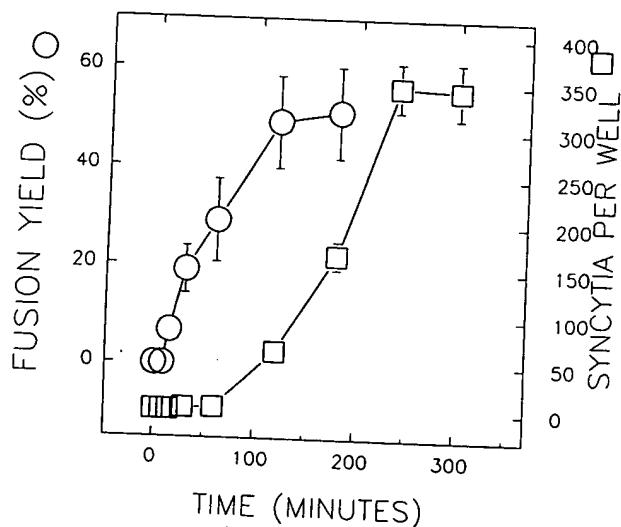
It was found that at high sCD4 concentrations (20 or 2  $\mu\text{g/ml}$ ) the reaction was biphasic, with maximal binding achieved after 30 or 90 min, respectively. After longer incubation periods, a marked reduction in surface-bound sCD4 was seen, reflecting shedding of gp120-sCD4 complexes. However, at low sCD4 concentrations (especially 0.05  $\mu\text{g/ml}$ ), the kinetics of binding was very slow, and equilibrium was not achieved even after 6 h [17].

Since we found that the rate of binding of sCD4 to HIV-1-Env-expressing cells was concentration-dependent, it was important to determine how these different binding rates correlated with the capacity of sCD4 to block syncytia formation. In the experiment depicted in Fig. 1, different concentrations of sCD4 were either pre-incubated with 12E1 (gp120/41)+ cells (effectors) for 1 h prior to mixing them with CEM (CD4+) cells (targets), or were added to the effector/target mixtures at time 0, or at different times after the initiation of coculture. sCD4 concentrations were maintained during the pre-incubation and subsequent cultures. The percentage of syncytia formation inhibition was dependent both on the sCD4 concentration and the time that sCD4 was added to the effector cells. The 1 h pre-incubation improved blocking at sCD4 concentrations of 0.2 and 2  $\mu\text{g/ml}$ , while 20  $\mu\text{g/ml}$  sCD4 was equally effective when added at time 0. Interestingly, little or no syncytia formation inhibition was seen when the addition of sCD4 to culture was delayed to 1 h after mixing. After 2 h, no significant inhibition of syncytia formation occurred, even when 20  $\mu\text{g/ml}$  sCD4 was used.

The lack of syncytia inhibition following the post-incubation with high sCD4 concentrations could be explained once the kinetics of effector-to-target cell fusion had been studied. As shown in Fig. 2, cell fusion between gp120/41-expressing 12E1 cells and uninfected CD4+ cells (in the absence of sCD4), as monitored by fluorescent dye redistribution [20,21], was initiated at 15 min and completed by 90 min post-cell mixing. Therefore, it is clear that when fusion was completed, the cells were no longer sensitive to sCD4 blocking (Figs 1 and 2). Syncytia formation, which depends on the fusion process, is slower. Syncytia first appeared at 90 min and peaked at 4–5 h.

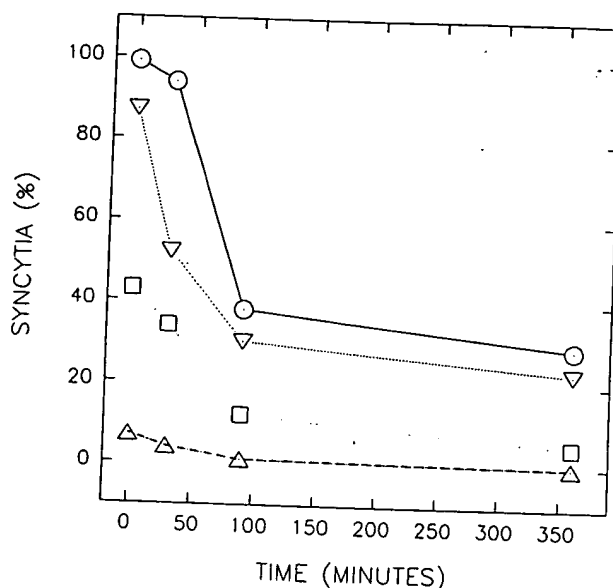


**Fig. 1.** Syncytia inhibition by soluble (s) CD4 depends on sCD4 concentrations and time of its addition to 12E1 (gp120/41) + effectors. 12E1 cells were infected with recombinant vaccinia vPE16 at 10 p.f.u./cell. Twenty hours later they were pre-incubated with no sCD4 (○) or concentrations of 0.2 µg/ml (●), 2 µg/ml (▽), or 20 µg/ml sCD4 (▼) for 1 h, followed by coculture with CD4+ CEM cells at 1:1 ratio ( $5 \times 10^4$  cells each) in 96-well plates, or sCD4 was added at the time of cell mixing (time 0), or at 30, 60, or 120 min following initiation of cocultures. Syncytia were scored after 5 h of coculture. Data are presented as percentages of control effector and target cocultures in the absence of sCD4.



**Fig. 2.** Kinetics of HIV-1-Env-mediated cell membrane fusion and syncytia formation. CD4- 12E1 cells were infected with vPE16 at 10 p.f.u./cell. Twenty hours later, effector cells were labeled with the lipid fluorophore Dil or the aqueous fluorescent dye BCECF (see Materials and methods). Labeled cells were mixed with CD4+ CEM target cells at a 1:1 ratio for syncytia formation, or at a 1:100 ratio, and monitored by video fluorescence microscopy to follow dye transfer between effectors and targets as a measurement of cell fusion [20]. Number of fused cells are expressed as percentages of total labeled effectors per culture (○), and number of syncytia, as mean + s.d. of triplicate cultures (□) at different time points after mixing of effectors and targets.

We then varied the duration of pre-incubation times with different sCD4 concentrations (Fig. 3). It was found that the inhibitory effect of sCD4 increased as a function of the pre-incubation time. However, with low sCD4 concentrations ( $< 0.2$  µg/ml), no complete blocking of syncytia formation was seen after 6 h pre-incubation. When compared with the binding data [17], this suggested that covering of 20–30% of surface gp120 molecules could result in 80% blocking of cell fusion. It is possible that, because of the oligomeric form of gp120/41 at the surface, binding of sCD4 to one gp120/41 molecule has a significant effect on the availability of other gp120/41 molecules in the oligomer to interact with surface CD4 on the target cells.



**Fig. 3.** Inhibition of syncytia formation by soluble (s) CD4 is dependent on sCD4 concentrations and on the length of pre-incubation. 12E1 (gp120/41) + effector cells were pre-incubated with sCD4 at 0.05 µg/ml (○), 0.2 µg/ml (▽), 2 µg/ml (□), or 20 µg/ml (Δ), for the indicated time periods, followed by their coculture with CEM cells at a 1:1 ratio, in the presence of identical sCD4 concentrations. Time 0, sCD4 was added at the time of effector/target mixing. Syncytia were scored after 5 h of coculture (in duplicate). Data are presented as percentages of control cultures (no sCD4).

#### Does gp120 shedding contribute to syncytia inhibition by sCD4?

It has previously been reported that when gp120/41-expressing cells or intact virions are incubated with sCD4 at high concentrations ( $> 5$  µg/ml), for 2–3 h at 37°C, displacement of CD4-gp120 from the surface occurs [7–12]. It was thus important for us to determine the extent of gp120 shedding and whether it leads to irreversible inhibition of syncytia mediated by Env-expressing cells.

sCD4-induced gp120 shedding was monitored in parallel by staining with FITC-OKT4 and polyclonal rabbit anti-gp120. As can be seen in Fig. 4, treatment of

12E1 (gp120/41)+ cells with 20  $\mu\text{g}/\text{ml}$  sCD4 for 3 h resulted in a marked reduction in their surface staining with FITC-OKT4 when compared with cells pre-incubated with sCD4 for 10 min only (Fig. 4a). This reduction was due to shedding of surface gp120, as confirmed by staining with the polyclonal rabbit anti-gp120 serum (Fig. 4b). We calculated that, after shedding, the cells retained between  $0.1\text{--}0.6 \times 10^4$  gp120 molecules per cell. In other studies, it was found that lower concentrations of sCD4 (2  $\mu\text{g}/\text{ml}$ ) can also induce shedding, but that they require longer incubation periods [17].

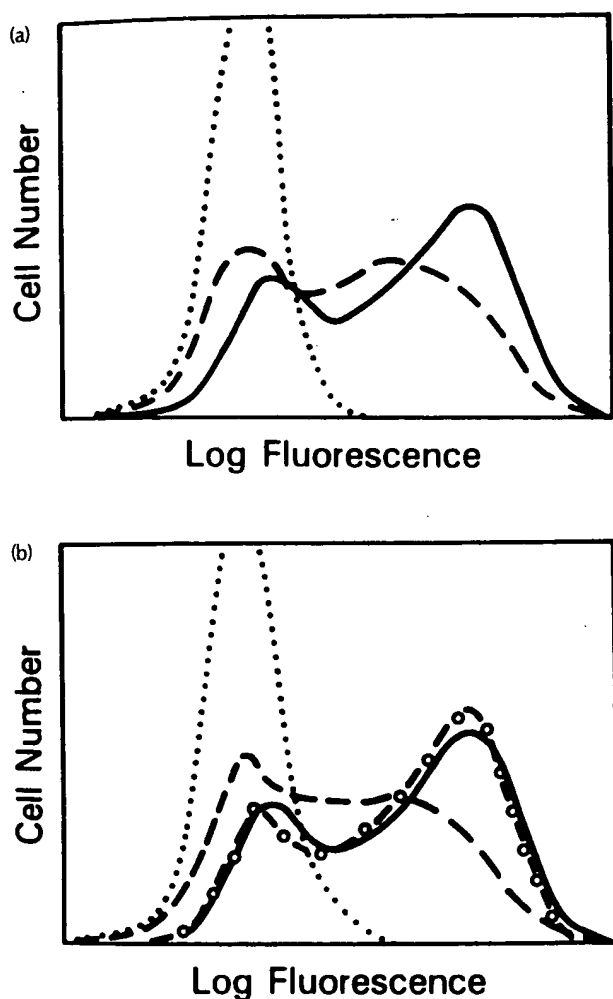
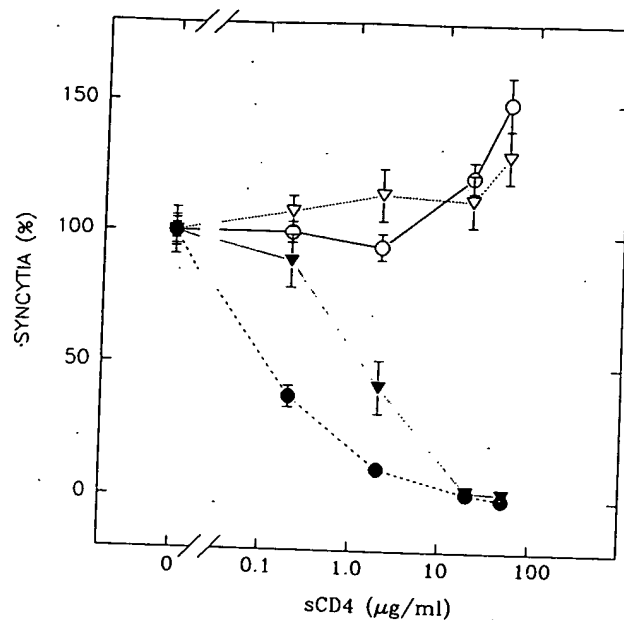


Fig. 4. Soluble (s) CD4-induced shedding of gp120 from 12E1 (gp120/41)-expressing cells. 12E1 cells were infected with recombinant vaccinia vPE16 at 10 p.f.u./cell. Twenty hours later, they were incubated with sCD4 at 20  $\mu\text{g}/\text{ml}$  for 10 (—) or 180 min (---). Cells were washed three times. (a) Cells stained with fluorescein isothiocyanate (FITC)-OKT4. (b) Cells stained with rabbit anti-gp120 followed by FITC-goat anti-rabbit at time 0 (○), or after 10 min (—), or 180 min (---) incubation with sCD4. (.....), background staining of untreated cells with FITC-goat anti-rabbit.

In order to assess the contribution of sCD4 binding versus sCD4-induced gp120 shedding to syncytia inhibition, we pre-incubated 12E1 (gp120/41)+ cells with different concentrations of sCD4 under conditions which allowed either maximal binding (30 min at 37°C), or binding and shedding (150 min at 37°C). The cells were then cocultured with CEM cells in the presence of identical sCD4 concentrations, or were first washed three times to remove unbound sCD4, and then mixed with CEM cells. Fifty per cent of surface gp120 molecules were shed after a 150 min incubation, and we expected that, even after washing of cells, a significant inhibition in either the number of syncytia formed and/or the rate of syncytia formation would take place. Data in Fig. 5 demonstrate that, in the continuous presence of sCD4, inhibition of syncytia formation was more substantial after 150 compared with 30 min pre-incubation with sCD4. Surprisingly, we found that, after washing of the pre-incubated cells, no syncytia formation blocking was seen, irrespective of whether the cells were pre-incubated for 30 or 150 min. Moreover, a 50% enhancement of syncytia formation was observed when cells were pre-incubated with 20  $\mu\text{g}/\text{ml}$  sCD4 for 150 min. Since 50% shedding of surface gp120 occurred, the lack of inhibition after washing of effector cells and removal of unbound sCD4 was unexpected. It was possible that the 12E1 cells which were infected with vPE16 at 10 p.f.u./cell, expressed gp120/41 at surface concentrations that were too high, so that even following incubation with high concentrations of sCD4 (20  $\mu\text{g}/\text{ml}$ ), not all the Env molecules were covered. We therefore repeated this experiment with 12E1 cells infected with vPE16 at either 10 or 1 p.f.u./cell. It was found that cells infected with 1 p.f.u./cell produced less syncytia compared with those infected with 10 p.f.u./cell (95 versus 225 syncytia per culture, respectively). Cells pre-incubated with sCD4 for 150 min and washed prior to culture, were not blocked at all. Instead, a small (50%) enhancement of syncytia formation was seen in the effector cultures infected with 1 p.f.u./cell vPE16 (data not shown).

Although the number of syncytia were not reduced, it was conceivable that the kinetics of syncytia formation were delayed due to bound sCD4 and/or shedding of surface gp120 molecules. We therefore investigated the kinetics of syncytia formation by cells pre-incubated for 5 h with no or increasing concentrations of sCD4 which were washed prior to mixing with CEM cells. It was found that both the total numbers of syncytia formed and the rate of syncytia formation were indistinguishable in all the cocultures.

Our data therefore suggest that: (1) shedding of gp120 molecules from cells as a consequence of incubation with sCD4 does not result in irreversible inhibition of their subsequent potential to fuse with CD4+ cells, and (2) bound CD4 molecules may not stay at the



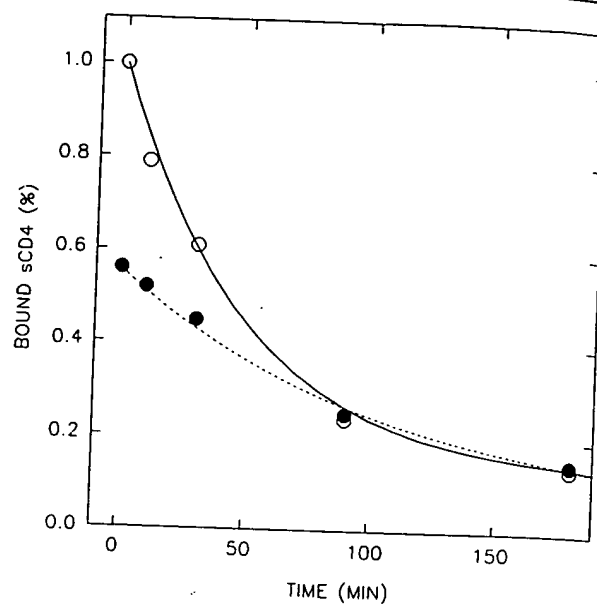
**Fig. 5.** Syncytia formation is not inhibited by soluble (s) CD4 pre-incubation, if the treated effectors are washed and cocultured with targets in the absence of sCD4. 12E1 cells were infected with vPE16 at 10 p.f.u./cell. Twenty hours later, they were pre-incubated with sCD4 (20  $\mu$ g/ml) for 30 ( $\nabla$ ,  $\blacktriangledown$ ), or 150 min ( $\circ$ ,  $\bullet$ ). Pre-treated effectors were either cocultured with CEM targets in the continuous presence of sCD4 (20  $\mu$ g/ml;  $\bullet$ ,  $\blacktriangledown$ ) or washed three times and mixed with CEM cells in the absence of additional sCD4 ( $\circ$ ,  $\nabla$ ). Data are presented as percentages of syncytia formed in control cultures (no sCD4 treatment).

surface of cells after removal of excess unbound CD4 long enough to block cell fusion. We examined this possibility by direct measurement of the dissociation of bound sCD4.

#### Kinetics of sCD4 dissociation

In order to directly measure the rate of dissociation of bound sCD4 from the cell surface, we examined cells under two experimental conditions which mimic the conditions used for the syncytia inhibition experiments described above. 12E1 (gp120/41)+ cells were pre-incubated with 20  $\mu$ g/ml sCD4 for either 10 (binding) or 180 min (binding and shedding). The cells were then washed three times and incubated in 1 ml RPMI medium supplemented with 10% FCS for an additional 150 min. At different intervals, the cells were stained with either FITC-OKT4 to monitor bound sCD4, or with rabbit anti-gp120 polyclonal serum to measure surface gp120.

As shown in Fig. 6, cells pre-incubated for 150 min with sCD4 (20  $\mu$ g/ml), shed approximately 45% of their surface gp120 compared with cells incubated for only 10 min (Fig. 6:  $\bullet$  versus  $\circ$  at time 0 of dissociation). After removal of excess unbound sCD4, a rapid decline in the surface-bound CD4 to either cell population was seen, so that after 90 min only approximately 12% of the initially-bound sCD4 molecules remained at the surface. We calculated the rate constants describing the sCD4 dissociation kinetics to be  $1.9 \times 10^{-2}$  and  $0.97 \times 10^{-2}$  per min for the cells pre-



**Fig. 6.** Kinetics of soluble (s) CD4 dissociation from gp120/41-expressing cells. 12E1 cells were infected with gp120/41-expressing recombinant vaccinia, vPE16 at 10 p.f.u./cell. Twenty hours later, cells were pre-incubated with sCD4 (20  $\mu$ g/ml) for either 10 (binding conditions;  $\circ$ ) or 150 min (binding and shedding conditions;  $\bullet$ ). Cells were then washed three times to remove unbound sCD4, and were incubated in 1 ml RPMI medium plus 10% fetal calf serum at 37°C in a CO<sub>2</sub> incubator with intermittent shaking. At the indicated time points, cells were stained with fluorescein isothiocyanate (FITC)-OKT4 to follow dissociation of sCD4 from their surface. Data are presented as ratios of the maximum bound sCD4 [after 10 min pre-incubation with 20  $\mu$ g/ml at 37°C, represented by time 0 ( $\circ$ )]. Lines represent least square fits to the experimental data by a single exponential dependence.

incubated with sCD4 for 10 and 180 min, respectively (Table 1). These dissociation rate constants are in agreement with the rates calculated from the sCD4 binding assays described previously [17]. Since the fusion process peaks at 90 min (Fig. 2), it is clear that the dissociating sCD4 leaves gp120/41 molecules accessible for fusion. Our results suggest that even after binding of sCD4 to cells under saturation conditions, once the unbound sCD4 concentration is significantly reduced, most surface-bound CD4 molecules dissociate at a faster rate than cell fusion occurs. This predicts an inefficient blocking of cell-to-cell virus transfer *in vivo* following administration of sCD4, unless very high sCD4 serum concentrations (> 20  $\mu$ g/ml) can be maintained for prolonged periods.

**Table 1.** Rate constants of soluble (s) CD4 dissociation from gp120/41-expressing cells.

sCD4 pre-incubation		Dissociation constants*		
Concentration ( $\mu$ g/ml)	Time (min)	A	B	k/min
20	10	0.88	0.12	$1.90 \times 10^{-2}$
20	180	0.50	0.06	$0.97 \times 10^{-2}$

\*The conditions of this experiment are as described in the legend to Fig. 6. The constants were calculated from the kinetic equation presented in Material and methods.

## Discussion

We followed in parallel: (1) the kinetics of sCD4 binding to gp120/41-expressing-cells as a function of sCD4 concentration; (2) the kinetics of syncytia inhibition when effector cells are incubated with sCD4 under the same conditions; (3) the contribution of sCD4-induced shedding of surface gp120 to subsequent syncytia inhibition; and (4) the dissociation rate of cell-bound sCD4 after removal of excess unbound sCD4.

We have determined previously [17] that the kinetics of sCD4 binding to HIV-1-Env-expressing cells was very slow at low sCD4 concentrations ( $<0.2 \mu\text{g/ml}$ ). Equilibrium was reached after 5 h, but not more than 25% of the surface gp120 molecules were sCD4 bound. At high sCD4 concentrations ( $2\text{--}20 \mu\text{g/ml}$ ), very fast binding occurred, peaking between 10–30 min, respectively. After longer incubation times ( $>3$  h), shedding of surface gp120 was induced by these sCD4 concentrations. However, even after 4–6 h, shedding did not exceed 50–70% reduction in surface gp120 molecules. Thus, under shedding conditions, the cell population expressed gp120 at intermediate to low levels, averaging  $0.1\text{--}0.6 \times 10^4$  molecules per cell.

Syncytia formation inhibition, following sCD4 pre-incubation, increased with increasing sCD4 concentrations and incubation times when sCD4 was maintained throughout the coculture period. Thus, at low sCD4 concentrations, prolonged pre-incubation times were required to obtain a maximal inhibition of 70–80%, whereas higher concentrations caused 100% inhibition and required shorter pre-incubation times.

However, if the gp120/41-expressing effectors were washed several times after pre-incubation with sCD4, no inhibition in either number or rate of syncytia formation was seen, irrespective of the sCD4 concentration used during the pre-incubation period. This was surprising since we established that, after several hours pre-incubation with high concentrations of sCD4, as many as 50–70% of the surface gp120 molecules were released. These findings suggest that shedding may not be an important mechanism leading to irreversible blocking of cell-to-cell virus transmission, as has previously been suggested for cell-free virions [9,10,22]. The reason for the differing results may relate to the fact that in these studies, shedding of gp120 from virions was induced. HIV-1 virions express 72 gp160 spikes ( $<300$  gp120 molecules per virion) [23], therefore, shedding of 50% of them may result in a critical reduction in the fusogenic ability of the virion [24]. Furthermore, many viral stocks were reported to spontaneously shed their surface gp120 molecules resulting in reduced infectivity titers [25]. It is obvious that the number of gp120 molecules expressed by infected cells (similar to our model system) is much higher.

Hence, even a substantial shedding is unlikely to be of much consequence in terms of subsequent cell-to-cell interactions and viral transmission. In several experiments, low levels of syncytia formation enhancement was observed after incubation with sCD4 under shedding conditions and subsequent washing of unbound sCD4. These findings are in agreement with recent reports on sCD4-mediated enhancement of simian immunodeficiency virus and HIV-2 infection, and syncytia formation [26,27]. It was postulated that the sCD4 pre-incubation may result in exposure of the gp41 fusogenic region making these cells more 'primed' for fusion [12,26]. However, we found that, even when cells were incubated with sCD4 under saturation but not shedding conditions and then washed, no syncytia formation inhibition was observed. This was found to be due to rapid dissociation of the surface-bound sCD4 molecules which was initiated as soon as the unbound excess sCD4 was removed. The rate of dissociation was faster than the rate of fusion. Thus, under conditions of rapid decline in free sCD4 concentrations (as in the blood stream shortly after administration of bolus sCD4), the previously bound molecules can dissociate rapidly resulting in minimal or no effect on cell-to-cell viral transmission.

The reversion of neutralization of syncytium inhibition is in agreement with our recent study, in which we demonstrated reversion of sCD4-mediated neutralization of intact virions [28]. Thus, the rapid dissociation of sCD4 takes place at the surface of both infected cells and free virions. The main difference between our studies and previous studies, which demonstrated very efficient sCD4-mediated blocking of HIV-1 infection and syncytia formation, is that in these previous studies sCD4 was maintained both during the pre-incubation and the coculture periods. Even in experiments in which the sCD4 was added post-viral binding at  $4^\circ\text{C}$ , the fusion process took place at  $37^\circ\text{C}$  in the presence of sCD4 [29]. Similarly, in shedding experiments, pre-treated virions or cells were cocultured with target cells in the continuous presence of sCD4 [9,10].

In summary, we demonstrate that sCD4 binding to cells is a reversible process which is very sensitive to fluctuations in sCD4 concentrations. *In vivo*, sCD4 has to compete with cellular CD4 receptors which are present at a constant high concentration. It is therefore not surprising that sCD4 was found to be ineffective in blocking or slowing the spread of HIV-1 infection.

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# Modulation of cell surface molecules during HIV-1 infection of H9 cells.

## An immunoelectron microscopic study

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**Objective:** To study cell surface molecules and HIV-1 proteins on H9 cells 2 days after infection by immunogold electron microscopy, either in single or in double labelling using combinations of host cell-derived molecules and HIV-1 proteins.

**Design and methods:** The presence of host cell antigens CD3, CD4 and human leukocyte antigen-DR (HLA-DR) and HIV-1 antigens gag p15, p17, p24 and env gp41 was evaluated using immunocytochemistry at the light microscopic level. H9 cells 2 days after infection were processed for conventional transmission electron microscopy and cryo-ultramicrotomy. Leukocyte antigens investigated were CD2, CD3, CD4 (two antibodies), CD5, CD8, CD25, CD30, CD63 antigens and HLA-DR; HIV-1-encoded antigens were gag p24, pol reverse transcriptase, and env gp41 and gp120. Double immunogold labelling was performed using reagents with different sized gold particles. For leukocyte markers, the labelling density of the cell membrane was assessed quantitatively on uninfected and infected H9 cells.

**Results:** Infected cells revealed the presence of gag p24, pol, and env gp41 and gp120 antigens on HIV-1 virions. Uninfected H9 cells showed a random distribution of cell surface molecules, including CD4 antigen, along the plasma membrane. The CD63 antigen, a lysosomal membrane glycoprotein, was located mainly in the cytoplasm of uninfected cells. Cells 2 days after infection showed CD4 labelling on sites where virions were budding from or attached to the cell surface and on free virions. Virions also showed labelling by CD3, CD5, CD25, CD30 and CD63 antibodies and anti-HLA-DR. Compared with uninfected cells, a significantly lower density was found on infected cells in labelling for CD4, CD5 and anti-HLA-DR. A significantly higher density on cells 2 days after infection was seen in CD63 labelling.

**Conclusion:** During the first phase of infection host cell molecules concentrate on budding structures and newly generated HIV-1 virions. This phenomenon might contribute to the disappearance of these molecules (like the CD4 molecule) from the cell membrane after infection.

AIDS 1992, 6:1105-1116

**Keywords:** CD4, HIV-1 antigens, H9 cells, immunocytochemistry, immunogold electron microscopy, *in vitro* infection, leukocyte surface molecules.

### Introduction

The CD4 molecule is one of the cell surface molecules involved in the infectious interaction between HIV-1

and host cells [1,2]. Other routes of infection are via the receptor for the Fc part of immunoglobulin [3,4] and via the receptor for the C3 component of complement [5]. A wide variety of cells, including

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T-lymphocytes, macrophages and antigen-presenting cells [1,2,6], can be infected via these routes.

HIV-1-infected cells manifest a down-regulation or density modulation of surface molecules. This has been demonstrated for the CD4 antigen [6-11], human leukocyte antigen (HLA) class I [12,13] and class II antigens [13,14], and the CD3, CD8 and CD11 antigens [15]. Proposed mechanisms to explain this phenomenon include down-regulation of corresponding mRNA [10]; loss of surface antigens to budding virions [12,13]; competitive masking of CD4 by viral envelope antigen [7]; and formation of complexes between the viral envelope glycoprotein and the CD4 receptor [7,10], followed by the internalization of the CD4 molecule with the viral component [9].

Methods for immunogold labelling of HIV-1-infected cells have been described previously [16-19]. This technique has the advantage of high electron density labelling, formation of stable complexes, and the simultaneous labelling of different antigens using gold particles of different sizes [20,21]. We have applied this technique to demonstrate HIV-1 antigens, cell surface markers and a lysosomal integral membrane protein [22,23] simultaneously. Our subcellular study of HIV-1-infected H9 cells was combined with immunocytochemistry at the light microscopical level. We observed a number of host cell molecules concentrated on budding virions.

## Materials and methods

### Virus and cells

H9 cells that were chronically infected or infected 2 days before harvesting with the HIV-1<sub>IIIb</sub> strain were used. Cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum and antibiotics at 37°C in humidified air containing 5% CO<sub>2</sub>. Freshly infected cultures were established by combining one part of infected cells with four parts of uninfected H9 cells. Uninfected H9 cells were used as control. Immunocytochemistry was performed on H9 cells that were either uninfected, chronically infected, or at 2 days after infection.

### Conventional transmission electron microscopy

This was performed according to conventional procedures. Briefly, a cell pellet was resuspended in 2% glutaraldehyde (GA) in 0.1 mol/l cacodylate buffer (pH 7.4) for at least 2 h at 4°C. After washing in the same buffer, the pellet was resuspended in human AB serum and centrifuged for 5 min. The supernatant was then removed and replaced by GA in cacodylate buffer. Fixation was performed for at least 24 h, followed by embedding in Epon. Ultrathin sections were contrasted with 6% uranyl magnesium acetate for 45 min

at 63°C, followed by Reynolds lead citrate for 10 min at room temperature.

### Immunoperoxidase labelling

The antibodies applied are listed in Table 1. Cyto-centrifuge preparations were fixed in acetone for 10 min at room temperature, and incubated in a three-step procedure with monoclonal antibody, rabbit antimouse immunoglobulin (Ig) conjugated to horseradish peroxidase, and swine antirabbit Ig conjugated to peroxidase (both conjugates from Dakopatts, Glostrup, Denmark). Primary antibodies were diluted in phosphate-buffered saline (PBS; pH 7.2) supplemented with 1% human serum albumin (Behringwerke, Marburg/Lahn, Germany). Secondary and tertiary antibodies were diluted in PBS with 10% heat-inactivated human AB serum. Washing between incubations was performed using PBS supplemented with 0.05% Tween 20. Peroxidase was visualized using H<sub>2</sub>O<sub>2</sub> and 3-amino-9-ethylcarbazole (AEC) as substrate. After fixation in PBS-buffered 10% paraformaldehyde (PFA) for 30 min, counterstaining was performed with haematoxylin. The optimal dilutions of each antibody were determined in introductory titration experiments. Control incubations included the omission of primary antibodies or replacement by an irrelevant one (for example, rabbit antimouse anti-serum or monoclonal antibody to human IgD of mouse IgG1 subclass). No labelling products were observed in these experiments.

### Immunogold labelling

Cells were pelleted and resuspended in 4% PFA in 0.1 mol/l phosphate buffer (pH 7.4) for 2 h at 4°C and washed twice in phosphate buffer supplemented with 0.15% glycine. The cell pellet was embedded in 10% gelatin. Cubes of this material were impregnated overnight at 4°C with 1.8 mol/l sucrose containing 15% poly(vinylpyrrolidone), mounted on copper holders and frozen in liquid nitrogen [24,25]. Ultrathin cryosections (80 nm) were cut at -110°C, thawed, and transferred to carbon Formvar-coated copper grids. In the immunolabelling procedure [26] the grids were incubated for 30 min with the first antibody directed to a cell surface molecule or to an HIV-1-encoded protein (at dilutions listed in Table 1). Depending on the type of antibody used, this first incubation was followed by a rabbit antimouse or antisheep Ig antibody (Dakopatts). The final incubation was performed using protein A-gold complex [26]. Complexes of 10 and 15 nm gold particles were used in most experiments. The second and third incubations were performed for 20 min and washed with PBS.

Double labelling of H9 cells infected for 2 days, for either HIV-1 antigen or CD3, CD4, or HLA-DR antigen, was performed as described by Slot *et al.* [26]. Ultrathin cryosections were incubated with CD3, CD4 or anti-HLA-DR antibody, followed by 1:400 diluted rabbit antimouse Ig and protein A-gold complex of 15 nm

Table 1. Antibodies applied to this study.

Antibody	CD	Subclass	Source*	Reciprocal dilution†	Reactivity
Anti-Leu-5b	CD2	γ2a	BD	10	All T-cells
Anti-Leu-4	CD3	γ1	BD	100	All T-cells
Anti-Leu-3a	CD4	γ1	BD	10	T-helper phenotype
ADP 336	CD4	γ2a	MRC	100	T-helper phenotype
Anti-Leu-1	CD5	γ2a	BD	10	All peripheral T-cells
Anti-Leu-2a	CD8	γ1	BD	10	T-cytotoxic/suppressor phenotype
Anti-IL-2R	CD25	γ1	BD	10	Activated T-cells
Ber-H2	CD30	γ1	Dakopatts	10	Activated lymphoid cells
RUU-SP 5.15	CD63	γ1	MM	3000	Platelets, macrophages and granulocytes
Anti-HLA-DR		γ2a	BD	10	HLA-DR
31.42.19		γ1	Abbott		HIV-1 gag p15
12.11.4		γ1	Abbott		HIV-1 gag p17
31.89.8		γ1	Abbott		HIV-1 gag p17
Anti-P24		Polyclonal‡	Seromed	2000	HIV-1 gag p24
ADP 380		γ1	MRC	10	HIV-1 pol reverse transcriptase
10.15.64		γ2a	Abbott	50	HIV-1 env gp41
Anti-gp120		Polyclonal‡	Seromed	100	HIV-1 env gp120

CD, cluster of differentiation; IL-2R, interleukin-2 receptor; HLA, human leukocyte antigen. \*Sources: BD, Becton Dickinson, Mountain View, California, USA; MRC, Medical Research Council, London, UK; Dakopatts, Glostrup, Denmark; MM, M.J. Metzelaar, PhD, (see [22,23]) Department of Haematology, University Hospital, Utrecht, The Netherlands; Seromed, Biochrom KG, Berlin, Germany; Abbott Laboratories, Diagnostic Division, North Chicago, Illinois, USA. †The reciprocal dilution used in immunogold electron microscopy is shown. ‡The sheep polyclonal antibodies were absorbed to human tonsillar lymphocytes before use to prevent binding to human cellular constituents.

gold particles. To prevent binding of the protein-A gold complex used in the second labelling, cryosections were fixed for 10 min in 1% GA and washed thoroughly. Incubation was then performed using 1:1500 diluted sheep anti-HIV-1 p24 followed by 1:500 diluted rabbit anti-sheep and protein A-gold complex of 10 nm gold particles. The optimal dilutions for each antibody were determined in introductory titration experiments. Controls included incubation of the anti-HIV-1-antibody using uninfected H9 cells as substrate; in addition, primary or secondary antibodies were omitted or applied at higher dilutions in single- or double-labelling experiments. To exclude labelling as a result of non-specific Ig-isotype binding to virus particles, we used anti-Leu-5b (IgG2a, CD2) and anti-Leu-2a (IgG1, CD8), which do not label H9 cells. No labelling product was observed in these control experiments.

The cryosections were embedded and contrasted in 1.8% methyl-cellulose and 3% uranyl acetate (pH 7.0), and examined using a Jeol 1200 EX electron microscope.

#### Quantitative measurements

The density of labelling products for CD3, CD4, CD5, CD25, CD63 and anti-HLA-DR antibodies on uninfected H9 cells and cells 2 days after infection was scored as follows. Representative cell surface segments of individual cells were selected at random. The number of grains was counted at magnification ( $\times 10\,000$ ), after visualization of the image on a television screen, and measurement of the length of the segment. Each measurement included the counting of a cell membrane of approximately 120  $\mu\text{m}$ , taken from

approximately 65 individual cells. Data were expressed as grains per 100  $\mu\text{m}$  cell membrane segment.

#### Statistical analysis

Analysis of variance was performed using SPSS PC3.0 software.

## Results

#### Light microscopic immunocytochemistry

H9 cells that were chronically infected or infected 2 days before harvesting were labelled by antibodies CD3, CD4, anti-HLA-DR, and anti-HIV-1 gag p15, p17, p24 and env gp41. The labelling by anti-gag antigens was cytoplasmic (Fig. 1a), whereas that by anti-env gp41, CD3 and CD4 was restricted to the cell surface. Uninfected H9 cells were not labelled by anti-HIV-1 antibodies. Labelling by the CD4 antibodies anti-Leu-3a and ADP 336 on chronically infected cells was of similar intensity, but less clustered than that on non-infected cells. CD4 labelling was also observed in-between cells (Fig. 1b). In contrast, only about 30% of H9 cells infected with HIV-1 2 days before harvesting were labelled by CD4 antibody, at a weak intensity.

#### (Immuno)electron microscopy

Conventional transmission electron microscopy of infected H9 cells revealed free and clustered virions, and virions (measuring 80–120 nm) attached to the cell surface. The virions showed electron-dense conical or bullet-shaped cores located centrosymmetrically or eccentrically in the capsule (Fig. 2a), as in previous reports [16,27,28]. Viral surface projections (spikes, representing env molecules) were observed occasionally

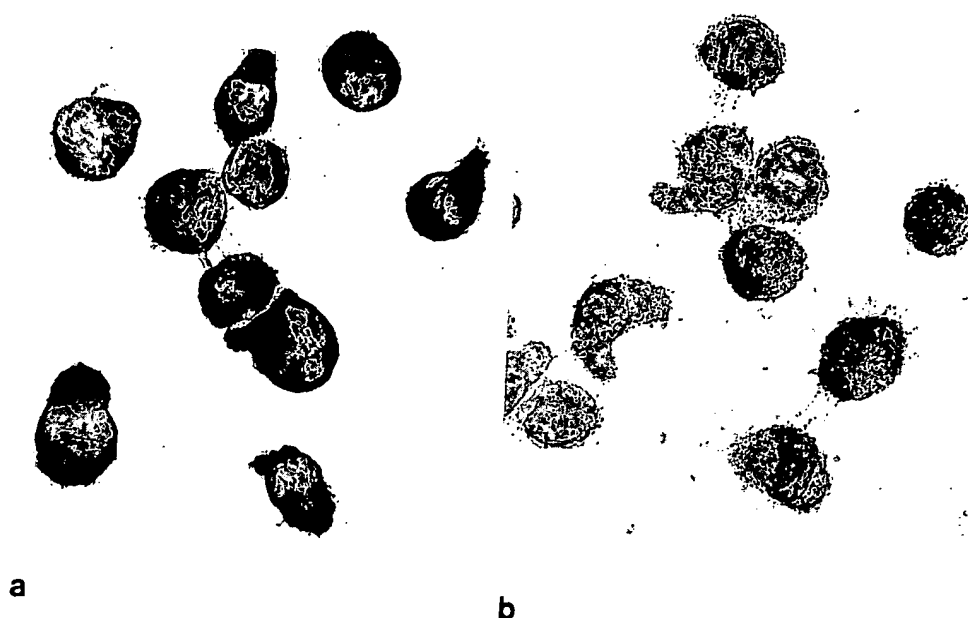


Fig. 1. Immunolabelling of H9 cells chronically infected with HIV-1 by (a) monoclonal anti-HIV-1 p24 antibody 31.89.8 and (b) CD4 antibody a-Leu-3a. Note the CD4 labelling patterns dotted on the cell surface and in-between cells. Three-step immunoperoxidase followed by AEC-detection and counterstaining by haematoxylin. Magnification,  $\times 730$ .

on free virions and budding profiles. Budding profiles were characterized by an electron-dense zone adjacent to humps in the host cell membrane. These typical characteristics of virions were lost in cryosections. Virions were easily identifiable using alternative morphological criteria (Fig. 2b).

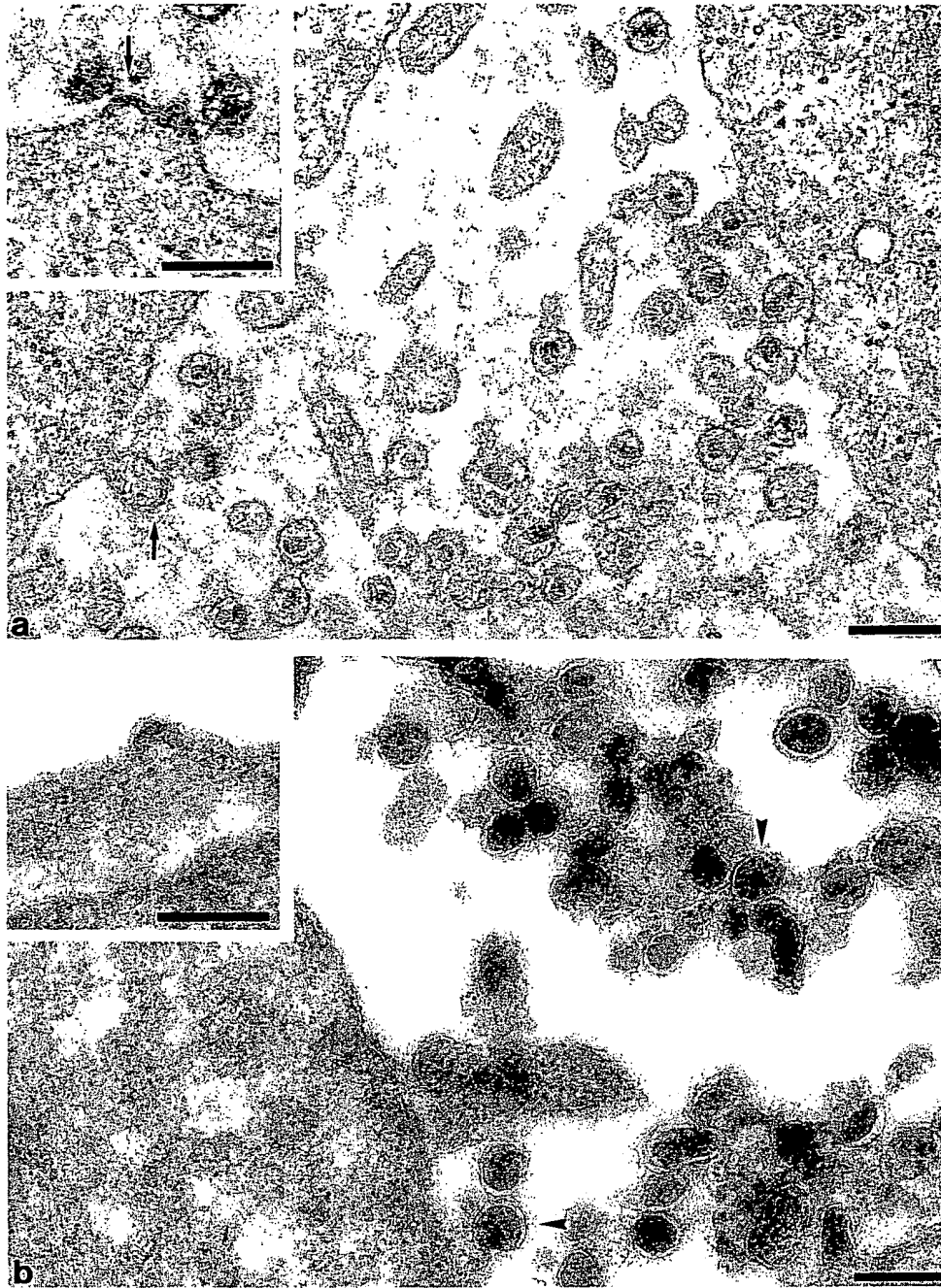
Murine monoclonal and sheep (polyclonal) antibodies to HIV-1 *gag* (Figs 3a–c), *pol* (Fig. 3d) and *env* (Fig. 4) antigens labelled the viral particles present in free form, in clusters in the extracellular space, and those that were attached to the cell surface. In addition, sites at the cell surface showed labelling where electron-dense aggregation indicated budding profiles (Figs 3b and 4b). The labelling product for anti-*gag* p24 (Figs 3a and b) and anti-*pol* (Fig. 3d) antibodies was observed mainly in virion cores, whereas labelling by anti-*env* gp41 and gp120 (Figs 4a–c) antibodies was restricted to virion peripheries. The anti-gp120 labelling product was also observed along the cell plasma membranes at 2 days after infection (Fig. 4d). Gold particles in anti-HIV-1 labelling were occasionally observed in the cytoplasm of infected cells (Fig. 3c).

Products of immunogold labelling of uninfected H9 cells by anti-HLA-DR (Fig. 5a), CD5 (Fig. 5c) and CD30 antibodies were abundant on the plasma membrane, and, in the case of CD30, in the cytoplasm. The immunogold labelling product of the CD4 antibodies anti-Leu-3a (Fig. 6a) and ADP 336 was observed mainly along the cell surface. On uninfected H9 cells, the labelling product of CD3 was distributed randomly along the plasma membrane, the nuclear membrane and in the cytoplasm. CD25 labelling produced a low density of gold particles on the cell membrane. The

label for CD63 (Fig. 6d) was associated mainly with vesicular or lysosomal structures in the cytoplasm, and little labelling product was found on the cell surface. Uninfected H9 cells showed no labelling for CD2, CD8 or HIV-1 antigens.

The surface of H9 cells 2 days after HIV-1 infection showed almost no labelling by CD4 (Fig. 6b) and a lower density of labelling by CD5 (Fig. 5d), CD30 and anti-HLA-DR (Fig. 5b) antibodies. The density of labelling product of CD3 and CD25 appeared similar to that on uninfected cells. An increase in density of the labelling product of CD63 along the plasma membrane of infected cells (Fig. 6e) was observed. Furthermore, CD3 (Fig. 7c), CD4 (Fig. 6c), CD5 (Fig. 5d), CD25, CD30, CD63 (Fig. 6f) and HLA-DR (Fig. 5b) labels were found on virion-like structures on or close to the plasma membrane and in the extracellular space between HIV-1-infected H9 cells. No labelling for CD2 and CD8 was observed.

Double immunogold labelling was performed using HIV-1 *gag* p24 and CD3, CD4 (anti-Leu-3a), or anti-HLA-DR. CD4 labelling on the plasma membrane co-localized to sites of HIV-1 virions identified by anti-p24 (Fig. 7b). This co-localization was also observed in double labelling in combinations with CD3 (Fig. 7c) and anti-HLA-DR. In addition, labelling product for CD3 (Fig. 7c) and anti-HLA-DR was present on the cell surface without co-localization with anti-p24, but at a lower density, as on non-infected H9 cells. Structures in the extracellular space labelled by CD3 (Fig. 7c), CD4 (Fig. 7a,b) and anti-HLA-DR showed co-expression with anti-p24.

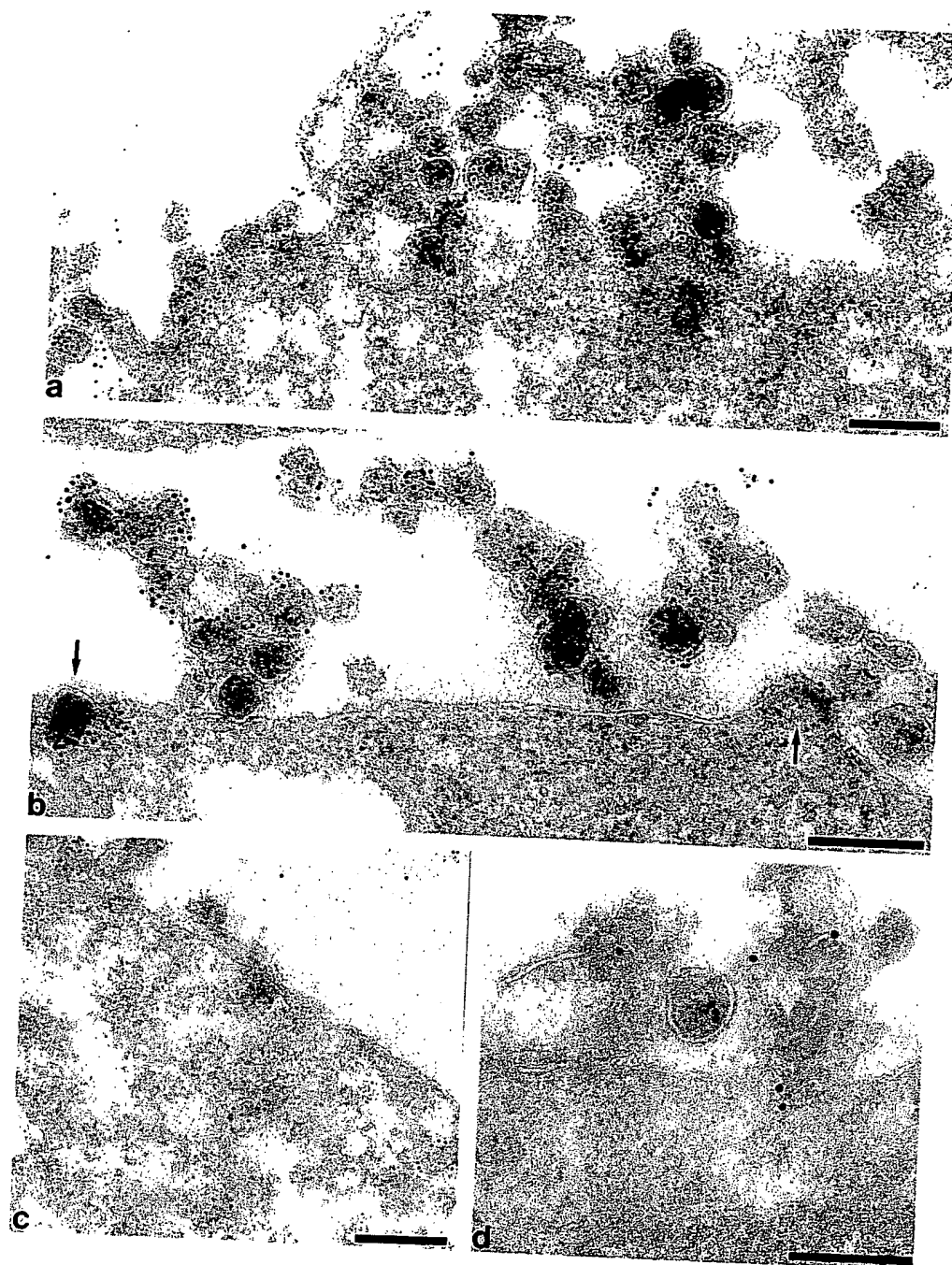


**Fig. 2.** Electron microscopy of a conventional plastic section [(a) and inset] and a cryosection [(b) and inset] of H9 cells 2 days after infection with HIV-1. (a) Shows virions approximately 80–120 nm wide with a dense, occasionally cone-shaped core, characteristic of HIV, and budding figures (arrows); dense patches adjacent to the plasma membrane. In cryosections (b), the ultrastructural retrovirus morphology is lost. Viral particles are recognized as round elements, usually more electron-dense than the cell cytoplasm, being enveloped by a membrane. Very few virions show a faint indication of a core (arrowheads). A budding figure [inset in (b)] is recognized as a dark extrusion from the cell surface, with a clear membrane. Bars represent 200 nm.

#### Quantitative measurements

The results of quantitative measurements of uninfected H9 cells and H9 cells 2 days after infection are presented in Fig. 8. For CD3 and CD25, the number of grains per 100  $\mu\text{m}$  membrane was similar for uninfected and infected cells. For both CD4 antibodies,

CD5 and anti-HLA-DR, the density was statistically significantly lower on infected than on uninfected cells. A lower density on infected cells was also observed for CD30, but this was not significantly lower than on uninfected cells. A significant increase in density on infected cells was observed for CD63.



**Fig. 3.** Immunogold electron microscopy of H9 cells 2 days after infection with HIV-1 (a, b and c) by polyclonal sheep anti-HIV-1 *gag* p24 antibody showing virion clusters that are densely labelled over the whole virion core and labelling of budding figures (arrows). There is also some intracellular labelling (c); (d) by monoclonal anti-HIV-1 *pol* reverse transcriptase antibody. Bars represent 200 nm.

## Discussion

We used immunogold electron microscopy to detect HIV-1 proteins in combination with host cell molecules on infected H9 cells. Appropriate controls excluded a possible non-specific labelling product by the antibodies applied. The location of HIV-1 proteins was in accordance with previous reports: *gag* proteins p15, p17 and p24 (Figs 3a and b) and *pol* reverse

transcriptase (Fig. 3d) occurred mainly in the core of virions or budding particles, and *env* proteins gp41 and gp120 (Figs 4a-c) in the envelope of the virus particles. The gp120 antigens were also detected on the cell membrane of infected cells (Fig. 4d). Except for reverse transcriptase, all HIV-1-encoded proteins were detected in the cytoplasm of infected cells. Thus, it is likely that the proteins detected represent newly synthesized viral components. The abundant expression

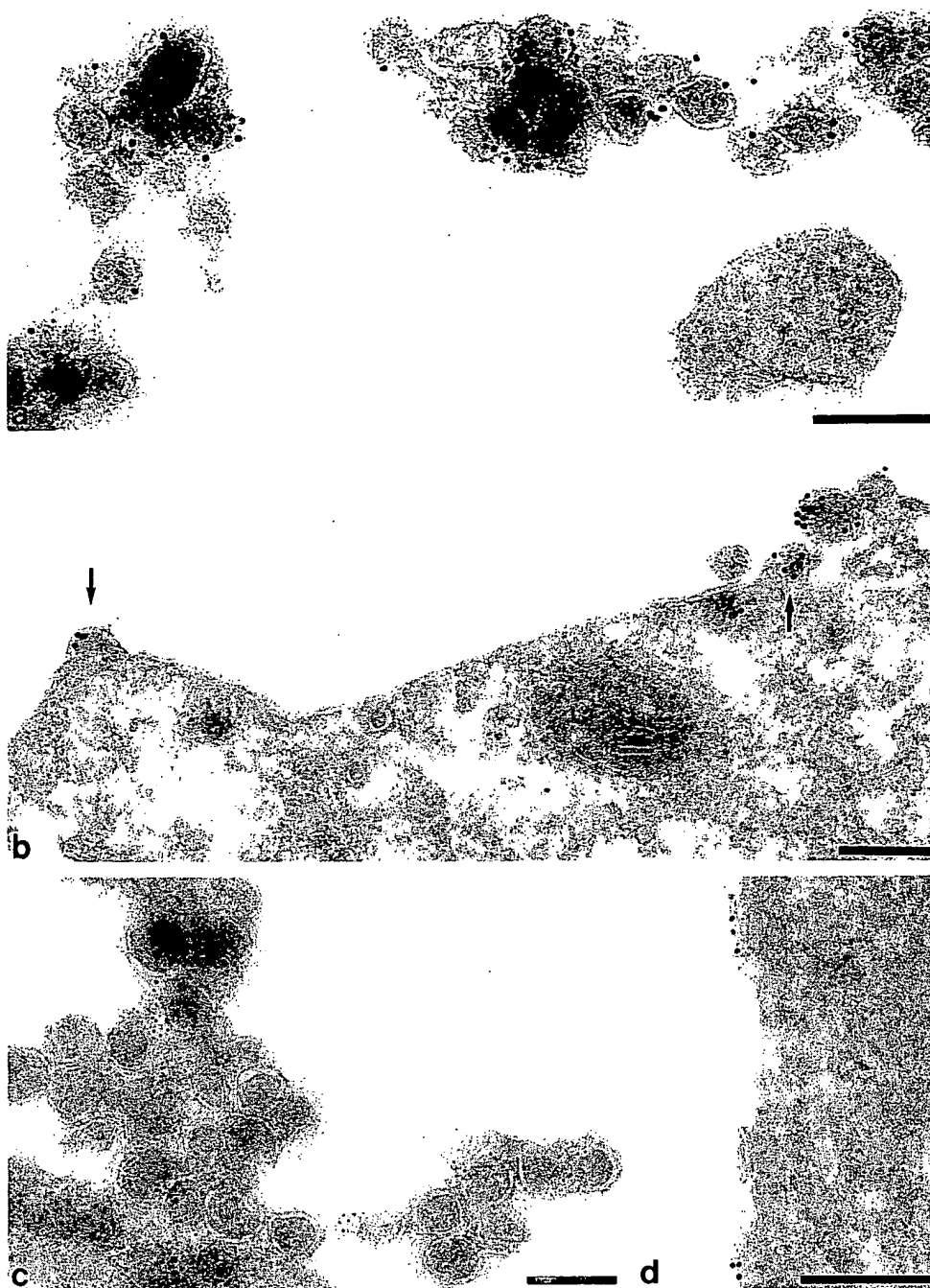


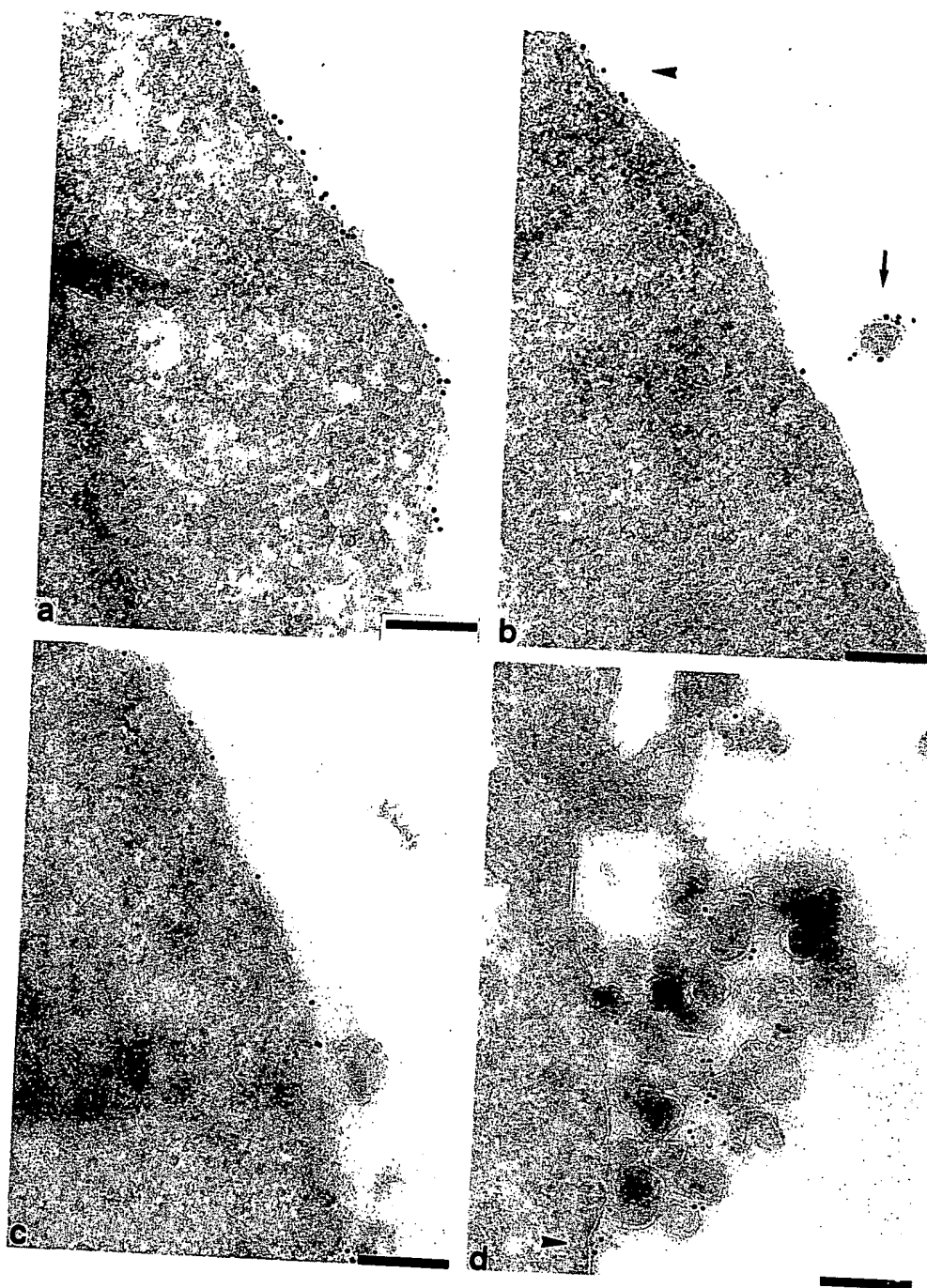
Fig. 4. Immunogold electron microscopy of H9 cells 2 days after infection with HIV-1 by (a and b) monoclonal anti-HIV-1 env gp41 antibody and (c and d) polyclonal sheep anti-HIV-1 env gp120 antibody. The labelling product of anti-gp41 is located mainly on the edge of virions (a) and budding figures (b); that for gp120 is observed on the envelope region of the virus (c) and on the plasma membrane of the cell (d). Bars represent 200 nm.

of HIV-1 mRNA (*in situ* hybridization; data not shown) and the presence of budding particles is in accordance with this suggestion.

The labelling of host cell molecules on H9 cells showed dramatic changes during infection. Light microscopic immunocytochemistry revealed that CD4 antigen had almost disappeared from cell membranes at 2 days after infection, but in chronically infected

cells it was localized on the cell membrane and in-between the cells. This pattern was examined more closely using immunogold electron microscopy. Here the CD4 molecule on cells at 2 days after infection was restricted to structures resembling budding structures and to free virions in-between the cells, and almost disappeared from the membrane of the H9 cell itself [illustrated in Figs 6b and c and (quantitatively) in Fig. 8]. This localization on HIV-1 virions was demonstrated

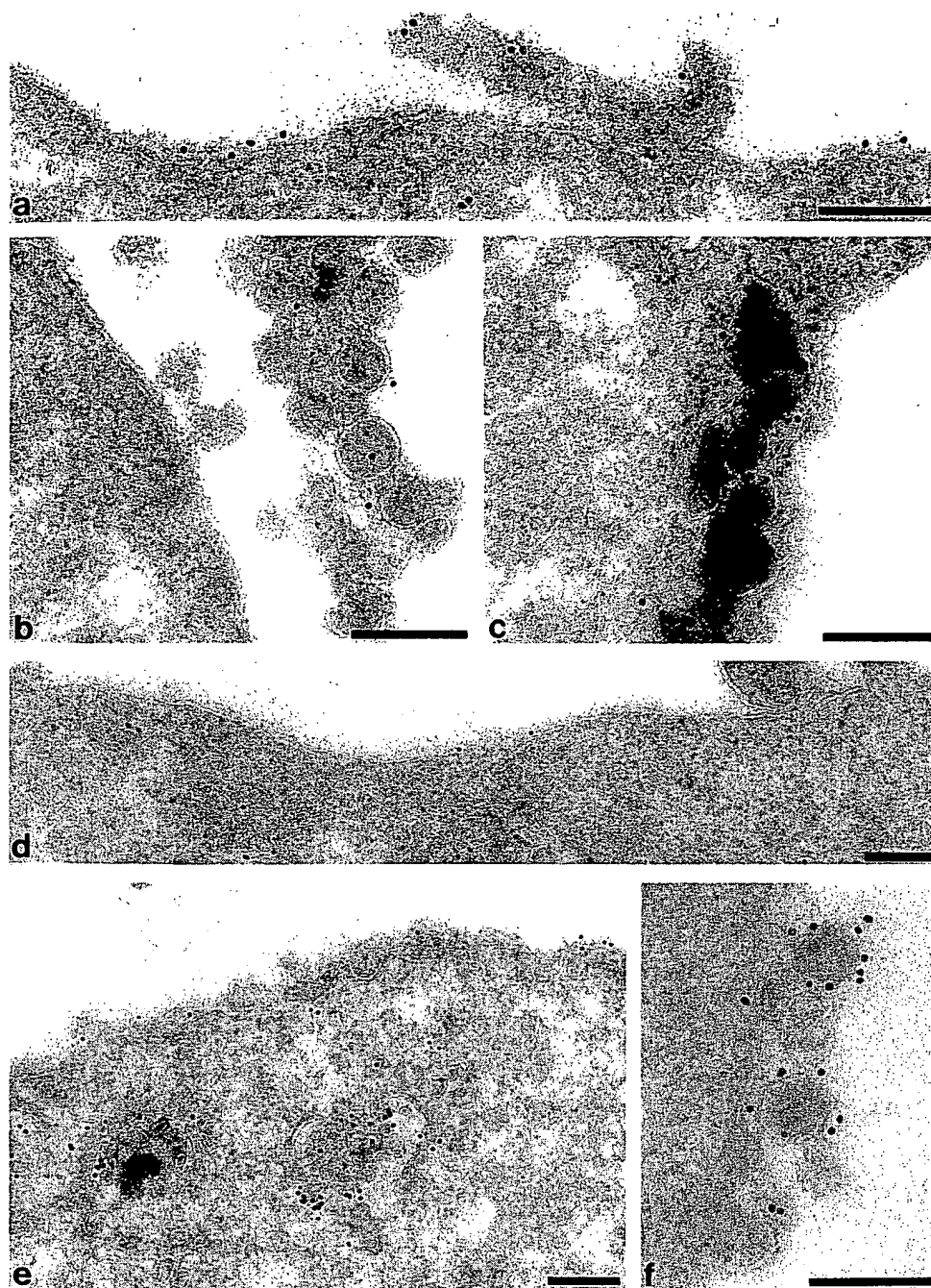




**Fig. 5.** Immunogold electron microscopy of (a and c) uninfected H9 cells and (b and d) H9 cells 2 days after infection by (a and b) anti-HLA-DR and (c and d) CD5 antibody. On uninfected cells the labelling product of anti-HLA-DR is densely located on the cell membrane (a), as is that of CD5. (c) On cells 2 days after infection, anti-HLA-DR label (b) occurs densely on a virus particle (arrow), and in low density on the cell surface (arrowheads), CD5 label (d) occurs on a cluster of virions and viruses still attached to the cell surface, and on the cell membrane (arrowhead). Bars represent 200 nm.

clearly using double immunogold labelling (Figs 7a and b). The CD4 molecule appears to be concentrated on the membrane of newly generated virions in the first few days after infection. A similar but less pronounced concentration was observed for HLA-DR (Fig. 5b) and CD5 antigen (Fig. 5d) that significantly decreased in density on the H9 cell membrane (except for their presence on virions). For CD3 (Fig.

7c) and CD25 antigens, the density on the H9 cell membrane remained similar after infection, and a significant increase was noted for CD63 (Figs 6e and f). All these antigens also appeared on virion membranes. The disappearance or decreased expression of hosts cell membrane molecules after HIV-1 infection (Fig. 8) has been described previously [7-15]: our data show that this phenomenon can be attributed to the concen-



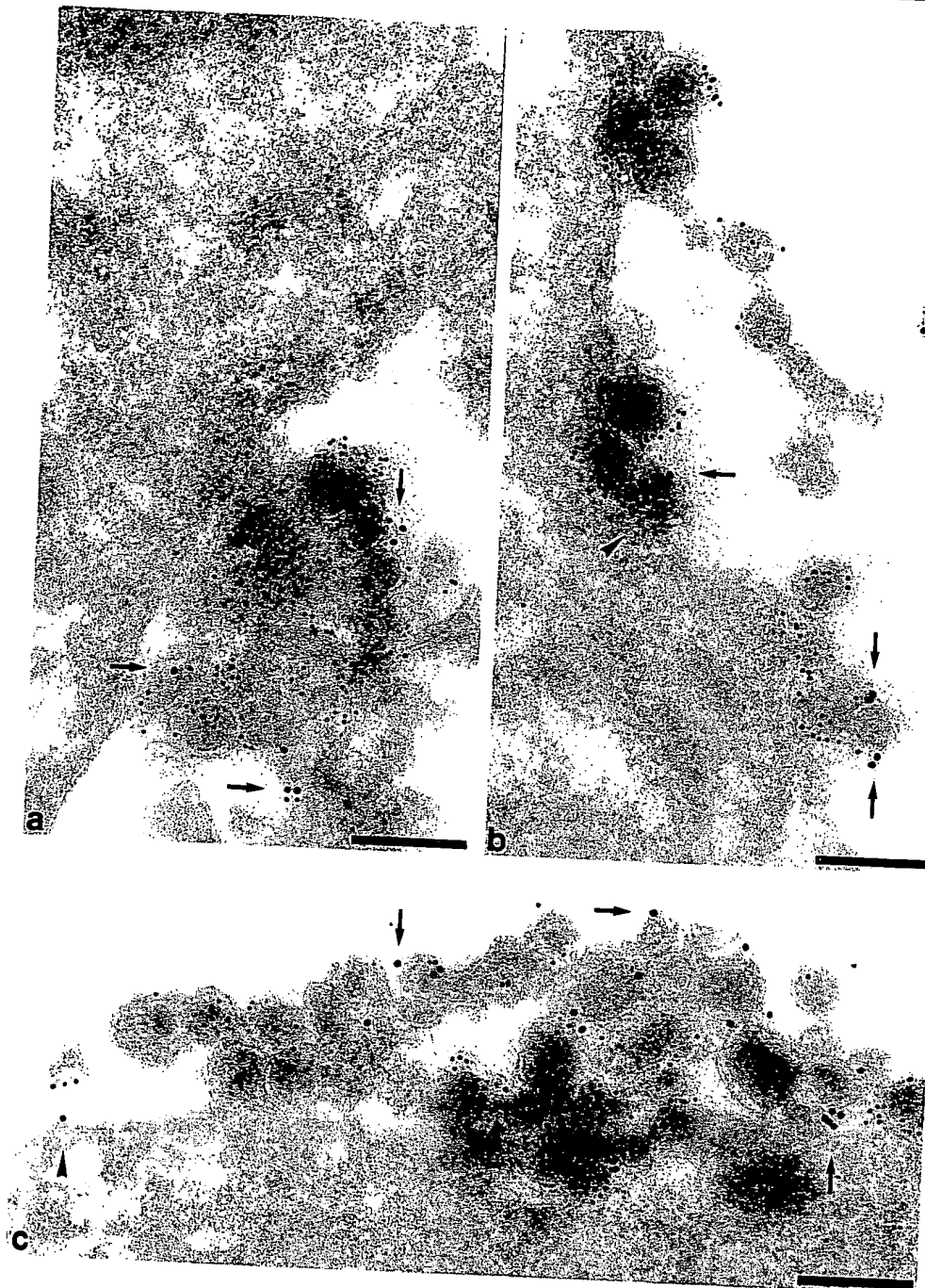
**Fig. 6.** Immunogold electron microscopy of (a and d) uninfected H9 cells and (b, c, e and f) H9 cells 2 days after infection by (a and b) CD4 antibody a-Leu-3a (c), CD4 antibody ADP 336 and (d–f) CD63 antibody. On uninfected cells the labelling product of CD4 is mainly on the cell membrane (a) and that of CD63 in the cytoplasm with some expression on the cell surface (d). On cells 2 days after infection, CD4 label is restricted to viral particles clustered outside the cell (b) or still attached to the cell membrane (c); CD63 label occurs in the cytoplasm (e), on the cell surface (e) and in high density on virions (f). Bars represent 200 nm.

tration of the molecules on budding-like figures and newly generated virions.

Other mechanisms underlying the disappearance of host molecules from the cell surface after infection may also be operating. These mechanisms include aspects of synthesis, for example, the decrease in

mRNA-encoding CD4 antigen [10], and catabolism, for example, the internalization of the molecule after binding viral components on the cell surface [9]. This phenomenon is most pronounced for the CD4 antigen, which almost completely disappeared from the cell membrane (Fig. 8). The combined effect of concentration on newly generated virions and decreased





**Fig. 7.** Double immunogold electron microscopy of H9 cells 2 days after infection, in the combinations (a and b) CD4 antibody a-Leu-3a (15 nm grains, arrows) and polyclonal anti-HIV-1 gag p24 (10 nm grains); and (c) CD3 (large grains, arrows) and polyclonal anti-HIV-1 gag p24 (small grains). The labelling product of CD4 occurs on viral clusters in the extracellular space, and occasionally on a structure resembling a budding figure [(b), arrowhead], but not on the cell membrane. CD3 antibody labels clusters of viral particles and can also be observed on the cell surface [(c) arrowhead]. These figures illustrate the co-localization of host cell molecules (CD4 and CD3 antigens) and virions (identified by p24 antibody). Bars represent 200 nm.

synthesis of new CD4 molecules results in insufficient molecules remaining to lodge freely in the cell membrane.

While concentration of host cell-derived molecules on budding structures and newly generated virions has been documented by others for HLA antigens [12,13], the underlying mechanism remains a matter

of speculation. It is logical to suggest that this mechanism is related to well-known interactions between host cell-derived and virally encoded molecules. This may concern the CD4 molecule and *env* gp120, but does not apply to the other molecules studied: a specific interaction with virus molecules has not yet been documented for the CD3, CD5, CD25, CD30 and CD63 molecules. It is tempting to speculate on an active in-

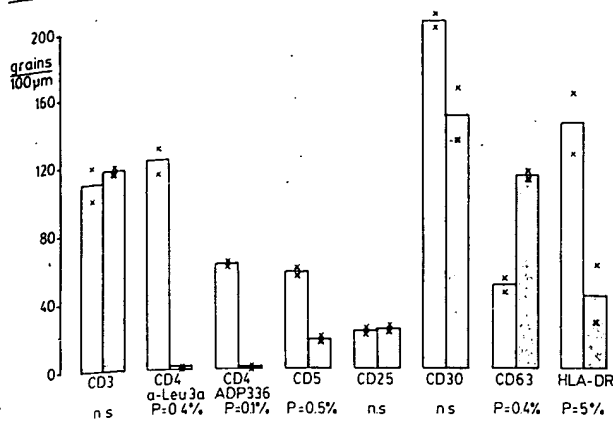


Fig. 8. Quantitative measurements of immunogold electron microscopy on cell membranes of uninfected H9 cells (□) and H9 cells 2 days after infection (■) for labelling by various antibodies to host cell surface molecules. Individual measurements are indicated (x). The statistical significance of difference between uninfected and infected H9 cells is shown for each antibody. Note the significantly lower density on infected cells for CD4 antibodies, CD5 and anti-HLA DR, and the significantly higher density on infected cells for CD63 antibody.

ternal metabolic pathway and cell membrane synthesis resulting in the movement of host cell membrane molecules to the site where new virions are budding. This may also apply to molecules that typically occur in the cytoplasm of uninfected cells, exemplified by the CD63 molecule (Figs 6e and f). This molecule is found on the cell membrane of cells after infection and on newly synthesized virions.

The presence of host-derived cell surface molecules on newly made virions is relevant to the route of infection of other cell types. These virions can use these host molecules for binding to cells in the first step of cellular infection. It remains to be established whether host-derived molecules are shed from the virion after budding and release, and how long a virion can use host-derived molecules in interaction with cells. In addition, the proposed hypothesis awaits confirmation by analysis of virus-producing cells after *in vivo* infection (taken from HIV-1-infected patients). Such studies have been initiated but hitherto have not given reliable results because of the low density of budding particles on cells from patients after *in vivo* infection.

In conclusion, we have shown that HIV-1 and its host cell share each other's molecules during the synthesis of new viral particles. HIV-1-encoded proteins occur on the host cell surface (gp120), but host-derived molecules concentrate on the viral envelope during budding and subsequent release. The presence of host-derived molecules on newly generated virions suggests that interactions between host molecules can play a role in the immediate spread of viral particles in the local environment of an organ. This is relevant both to preventing virus spread and to vaccine development.

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## Cell-to-Cell Spread of HIV-1 Occurs within Minutes and May Not Involve the Participation of Virus Particles

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Although virus infections have been classically studied with "cell-free" virion preparations, many animal viruses are able to spread both *in vitro* and *in vivo* by inducing cell-cell fusion. An efficient system to monitor the cell-to-cell spread of HIV-1 has been developed employing chronically infected H9 donor cells. Under appropriate conditions of cocultivation with uninfected cells, the synthesis of unintegrated viral DNA, monitored by Southern blot hybridization, occurred between 2 and 4 hr following infection; viral proteins were detected 8 to 12 hr following cocultivation and progeny virions were released into the medium by 16 hr. The use of metabolic inhibitors or specific envelope/receptor antibodies revealed that the cell-to-cell spread of HIV required: (1) gp120-CD4 interaction and (2) reverse transcription. Light and electron microscopy, fluorescent dye redistribution, and soluble CD4 competition experiments all demonstrated that the HIV-induced cell-cell fusion began within 10 to 30 min of cocultivation. Surprisingly, the electron microscopic analyses also suggested that budding or mature virus particles did not participate in this process. Thus the virus-induced cell-cell fusion observed is very likely the result of gp120/gp41 proteins, on the surface of infected cells, interacting with CD4 molecules on uninfected cells. These findings are of immediate importance in understanding the mechanism(s) of HIV-1 transmission *in vivo* and for the design of effective vaccines and antiviral agents. © 1992 Academic Press, Inc.

### INTRODUCTION

Cell-free virus preparations are the commonly accepted inocula used to initiate experimental laboratory infections of tissue cultures or susceptible animals. Many viruses, however, remain intimately associated with their host cells and appear to be transmitted, particularly *in vivo*, cell-to-cell rather than as cell-free particles. This is certainly the case for some herpesvirus infections in which virus spread has been shown to occur in the presence of neutralizing antibody (Black and Melnick, 1955; Hoggan and Roizman, 1959; Lodmell *et al.*, 1973). Cell-to-cell transmission has been described for several animal viruses including vaccinia, foamy virus, and especially paramyxoviruses (Enders and Peebles, 1954; Henle *et al.*, 1954; Okada, 1958; Johnson and Scott, 1964; Hooks *et al.*, 1976). Among the retroviruses, the human T-cell leukemia virus type I (HTLV-I) is generally considered to be a tightly cell-associated agent. Although some HTLV-I infections have been established with cell-free preparations (Clapham *et al.*, 1983), virus isolations as well as *de novo* infections are commonly effected by cocultivation with uninfected CD4<sup>+</sup> human T-cells, implying that cell-to-cell

spread may be the more important mode of transmission.

A puzzling feature of the human immunodeficiency virus type 1 (HIV-1) life cycle *in vivo* is that disease progression occurs despite the presence of low levels of cell-free virus in the plasma (Michaelis and Levy, 1987; Ho *et al.*, 1989; Coombs *et al.*, 1989; Venet *et al.*, 1991). It is possible that the chronic production of HIV in an infected individual, which can persist for many years, is primarily sustained by cell-to-cell virus transmission. It is also likely that infections are established *in vivo* following the transfer of infected cells between sexual partners or as a result of intravenous drug use. The potential importance of cell-to-cell virus spread is supported from tissue culture studies in which HIV-1 preparations, defective in *vif* gene function, are unable to initiate infections as cell-free particles yet retain the capacity to infect CD4<sup>+</sup> T-cells following cocultivation (Strebel *et al.*, 1987; Fisher *et al.*, 1987). In this paper, we report a human T-lymphocytic cell line system which models cell-to-cell HIV spread and exhibits very rapid infection kinetics. In this system, cell-cell fusion occurs within minutes following the cultivation of virus-infected donor cells with CD4<sup>+</sup> recipient cells. This process does not seem to require budding or mature virions. Inhibitor studies indicate that gp120-CD4 interaction and reverse transcription

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are required during the early steps of the cell-to-cell spread of HIV.

## MATERIALS AND METHODS

### Cells

The CD4-positive human T-lymphocytic cell line H9 (Mann *et al.*, 1989) was infected with HIV<sub>III</sub>B (Popovic *et al.*, 1984) and was maintained ( $5 \times 10^5$  cells/ml) in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal calf serum for more than 1 month. These chronically infected H9/HIV<sub>III</sub>B cells produced  $0.4\text{--}2 \times 10^6$   $^{32}\text{P}$  cpm/ml RT activity (Willey *et al.*, 1988) during a 48-hr period when seeded in fresh medium at a density of  $5 \times 10^5$  cells/ml. A2.01 cells (Folks *et al.*, 1986), a CD4<sup>+</sup> derivative of the A3.01 (Folks *et al.*, 1985), were used as recipients in some experiments.

### Cell-to-cell and cell-free HIV infections

The HIV<sub>III</sub>B producing H9 cells were seeded at  $5 \times 10^5$  cells/ml in 10 ml of RPMI 1640 supplemented with 10% fetal calf serum and cultivated for 48 hr. The culture was then transferred to a 50-ml polypropylene conical centrifuge tube and the H9/HIV<sub>III</sub>B cells were allowed to settle for 40 min at room temperature. Cells and supernatant (filtered through a 0.22- $\mu\text{m}$  filter and containing approximately  $2 \times 10^6$   $^{32}\text{P}$  cpm/ml RT activity) were collected and separately used to infect fresh cells. For the cell-to-cell infection, H9/HIV<sub>III</sub>B cells ( $1 \times 10^6$ ) were mixed with uninfected H9 cells ( $4 \times 10^6$ ) at time zero and cultivated in 10 ml of fresh medium at a density of  $5 \times 10^5$  cells/ml. For cell-free virus infection, uninfected H9 cells ( $4 \times 10^6$  cells) were suspended in 4 ml of the filtered supernatant at time zero, fresh medium was added at 8 and 48 hr postinfection, and the cultures were maintained at a density of  $5 \times 10^5$  cells/ml. In both infections, HIV replication was monitored by unintegrated viral DNA, viral protein, and virion production.

### Detection of unintegrated HIV viral DNA by Southern blot analysis

Low-molecular-weight DNA was prepared from infected cells by differential salt precipitation (Hirt, 1967). Approximately  $1 \times 10^6$  H9 cell aliquots were washed once with PBS, lysed in Hirt solution (10 mM EDTA, 100 mM Tris-HCl, pH 8.0, 0.6% SDS) for 30 min at room temperature, and incubated in 1 ml of Hirt solution containing 1 M NaCl at 4° overnight. The lysate was centrifuged at 17,000 *g* for 30 min and DNA purified from the supernatant was electrophoresed through a 0.6% agarose gel, transferred to a nitrocellu-

lose membrane, hybridized to  $^{32}\text{P}$ -labeled pBENNA4 (Folks *et al.*, 1985), an HIV recombinant plasmid DNA containing the 2.7-kb *EcoRI*–*Bam*HI segment from cloned pNL4-3 (Adachi *et al.*, 1986) viral DNA, and subjected to autoradiography.

### Detection of HIV proteins by Western blot analysis

Approximately  $1 \times 10^6$  cells were collected from the cultures, washed once with PBS, and lysed in CHAPS buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 100 mM NaCl, 0.5% CHAPS, and 0.2% deoxycholate) for 5 min on ice. The lysate was then centrifuged for 5 min at 17,000 *g* and the supernatant was subjected to Western blot analysis. The proteins (50  $\mu\text{g}$  protein per lane) were electrophoresed in a SDS–12% polyacrylamide gel, transferred to nitrocellulose membrane, blocked with 10% skim milk in TBS (10 mM Tris-HCl, pH 7.4, 140 mM NaCl), incubated with AIDS patient serum, probed with  $^{125}\text{I}$ -protein A, and subjected to autoradiography.

### Detection of virion production by reverse transcriptase assay

Supernatants of virus producing cultures were collected at different times following infection, and RT activity was measured using [ $^{32}\text{P}$ ]TTP as described previously (Willey *et al.*, 1988).

### Metabolic inhibitors or monoclonal antibodies

HIV<sub>III</sub>B chronically infected and uninfected H9 cells were cultured separately for 1 hr in medium containing various concentrations of metabolic inhibitor or monoclonal antibody. The two cultures were then mixed together at a ratio of 1:4 (infected:uninfected) and cultured in the presence of the same concentration of inhibitor/monoclonal antibody for 4 (analyses of unintegrated DNA) or 40 (RT assay) hr and maintained at a density of  $5 \times 10^5$  cells/ml. In some experiments, H9 cells ( $4 \times 10^5$  cells) were cultured in 1 ml of medium in the presence of increasing concentrations of inhibitor for 1 hr and then labeled with [ $^3\text{H}$ ]thymidine (48 Ci/mmol, 1  $\mu\text{Ci}$ /ml) for an additional 2 hr. The cells were then harvested, washed once with PBS, and denatured in 5% trichloroacetic acid at 0°. The incorporation of the thymidine into acid-insoluble material was determined by liquid scintillation counting.

### Cell-cell fusion monitored by light or electron microscopy

Cells ( $1 \times 10^7$ ) were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, at room temperature for 15 min followed by overnight at 4°. The fixed

cells were pelleted in a microfuge and gelled into cold agar. After postfixation in  $\text{OsO}_4$  and *en bloc* staining with uranyl acetate, they were dehydrated and embedded in Spurr's plastic. For light microscopic analyses, 1- $\mu\text{m}$  plastic sections were stained with toluidine blue. For electron microscopic analyses, the thin sections of the cells were stained with uranyl acetate and lead citrate and examined on a Zeiss EM10A electron microscope (Klimkait *et al.*, 1990).

#### Cell-cell fusion monitored by fluorescent dye redistribution

Chronically HIV-infected H9 cells ( $1 \times 10^6$ ) in 1 ml of serum-free RPMI 1640 medium were incubated with 10  $\mu\text{l}$  of 1 mM BCECF-AM, a water-soluble fluorescent dye, for 30 min at 37° in a  $\text{CO}_2$  incubator, washed once with serum-free RPMI 1640 medium, and resuspended in complete medium containing 10% heat-inactivated BSA at a final concentration of  $4 \times 10^5$  cells/ml. The labeled infected H9 cells (500 cells in 10  $\mu\text{l}$ ) were mixed with  $5 \times 10^4$  uninfected H9 or CD4-negative A2.01 cells in 0.1 ml in 96-well plates; samples were removed following 0, 10, 15, 30 or 60 min incubation at 37° and examined by fluorescent microscopy. Fluorescent cells (individual as well as higher order aggregates) were counted in three different fields of duplicate wells. The fusion yield was defined as the number of fluorescent cells present in aggregates (usually doublets and triplets) divided by the total number of labeled cells multiplied by 100.

#### Soluble CD4 competition analysis

Uninfected H9 cells ( $5 \times 10^4$  cells in 45  $\mu\text{l}$  medium) were mixed with H9/HIV<sub>III</sub>B cells ( $5 \times 10^3$  cells in 45  $\mu\text{l}$  medium) and 10  $\mu\text{l}$  of soluble CD4 (sCD4) (1 mg/ml) was slowly added without disturbing the cells at 0, 15, 30, or 60 min following cocultivation. Syncytia were counted 6, 30, and 92 hr following coculture. The fusion yield was defined as the number of syncytia formed in the presence of sCD4 divided by the number of syncytia present in its absence (96 and 110 syncytia detected at 6 and 92 hr, respectively) multiplied by 100.

#### Chemical agents

Dextran sulfate (MW5000), heparin (sodium salt grade I), phosphonoformate (PFA, trisodium salt), and aphidicolin were purchased from Sigma (USA). 3'-Azido-3'-deoxythymidine (AZT) was a generous gift of Dr. H. Mitsuya, National Cancer Institute. Dextran sulfate, heparin, PFA, and AZT were dissolved in PBS at 10 mM, and aphidicolin was dissolved in dimethyl sulfoxide at 10 mg/ml for stock solution.

## RESULTS

### Establishment of a tissue culture system to model the cell-to-cell spread of HIV

We began investigating the cell-to-cell spread of HIV by evaluating several "donor" cell systems capable of efficiently delivering virus to susceptible "recipient" cells. Our survey included chronically HIV-1 producing cells of the HUT78 (Gazdar *et al.*, 1980) and HeLa CD4<sup>+</sup> (Maddon *et al.*, 1986) lineages as well as an acutely infected human T-cell line [A3.01 (Folks *et al.*, 1985)], harvested near the peak of progeny virus production. Infections could be most rapidly and consistently established with virus producing H9 cells; efficient cell-to-cell transfer of the HIV infection to several different human T-cell lines was observed (data not shown). In these preliminary studies, we noted that the H9 cells could be enriched for cell-to-cell transmission capacity if they were allowed to "settle" to the bottom of a conical centrifuge tube for 40 min prior to their use as an inoculum to initiate virus infections. Trypan blue staining studies revealed that >95% of the "settled" cells were viable compared to only 30–50% of the H9 cells in the supernatant.

In the experiments to be described comparing cell-free and cell-to-cell virus infections, the inocula consisted of filtered supernatant (cell-free) or *one-fifth the number of infected cells* used to produce that supernatant (cell-to-cell). No attempt was made to normalize for RT activity nor to concentrate the cell-free virus inoculum. In these side-by-side comparisons, fresh, fractionated cells and supernatants were analyzed; however, the cell-free virus arm of the experiment was always in fivefold relative excess. Several parameters of *de novo* HIV infection were monitored including the synthesis of unintegrated viral DNA, the production of viral proteins, and the release of progeny virions into the medium. As shown in Fig. 1A, no viral DNA could be detected in the cocultures up to 2 hr following the incubation of uninfected H9 cells with the virus producing H9 cells at a ratio of 4:1. However, significant levels of unintegrated DNA were synthesized 2 to 4 hr following cocultivation and rose over the next 20 hr. This was in marked contrast to the companion infection inoculated with the cell-free virus generated from the same cells; unintegrated viral DNA was not detected until 48 to 72 hr postinfection and peaked on Day 4. By 8 hr following cocultivation, 400 pg, or approximately 40 copies of unintegrated HIV viral DNA per cell, was present in the cultures (Fig. 1A, left). It should be noted that the relatively insensitive technique of Southern blot hybridization (rather than polymerase chain reaction) was used to monitor the synthesis of viral DNA following cocultivation; the rapidity of

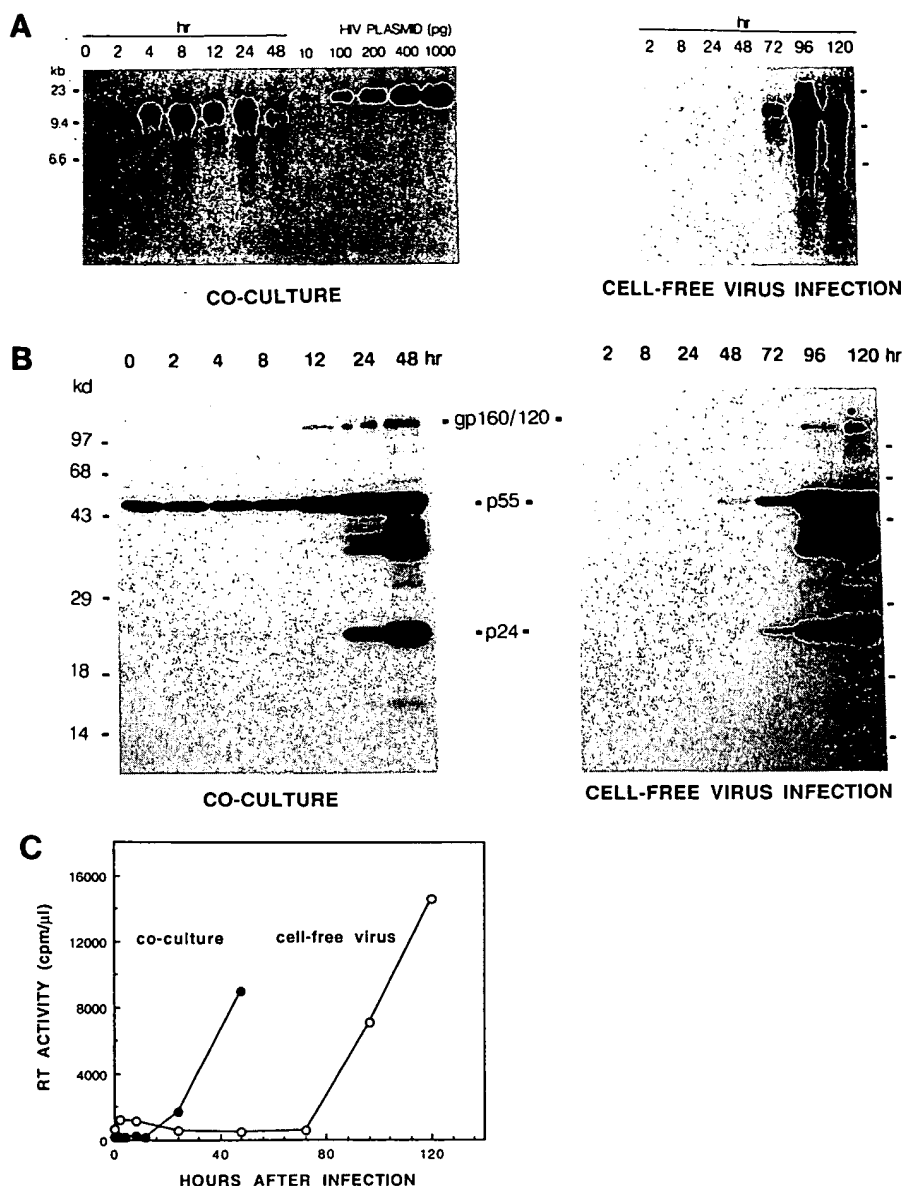


FIG. 1. Comparison of HIV-1 replication kinetics in cell-free and cell-to-cell infections. (A) Unintegrated viral DNA synthesis. Hirt DNAs were extracted from infected cells at the indicated time following infection and subjected to Southern blot analyses using a  $^{32}\text{P}$ -labeled HIV-specific probe. For quantitation of HIV viral DNA, increasing amounts of HIV plasmid pNL4-3, which contains a full-length HIV provirus, were analyzed in the same gel. (B) Viral protein synthesis. Cytoplasmic proteins were extracted from infected cells with CHAPS buffer at the indicated times following infection and subjected to Western blot analyses using AIDS patient's serum. (C) Progeny virus production. Supernatants of the two infected cultures were collected at the indicated times following infection and assayed for RT activity using  $^{32}\text{P}$ TTP. The RT activity produced in the parental H9/HIV<sub>III</sub> culture was simultaneously monitored during the experimental period and subtracted from that present in the coculture.

its accumulation and the high levels detected were thus that much more striking. The extremely rapid cell-to-cell spread of HIV was not limited to H9 recipient cells; similar kinetics of viral DNA accumulation was also observed when A3.01 recipient cells, a cloned derivative of CEM (Foley *et al.*, 1965) cells, were used (data not shown). Furthermore, similar amounts of viral DNA were rapidly synthesized when H9 cells, chronically producing HIV<sub>LAI</sub> (Wain-Hobson *et al.*, 1991) or

HIV<sub>NL4-3</sub> (Adachi *et al.*, 1986), were employed as inocula. No evidence of cell-to-cell HIV spread, as monitored by viral DNA synthesis, was detected when the CD4-negative derivative of A3.01 cells, designated A2.01 (Folks *et al.*, 1986), was used as the recipient (data not shown).

The kinetics of viral protein synthesis as a consequence of cell-to-cell or cell-free virus infection was compared by immunoblotting infected cell lysates. The

donor HIV<sub>III</sub> producing H9 cells synthesized substantial amounts of the p55 Gag precursor which was readily detected at the onset of cocultivation (Fig. 1B). Increased levels of p55 were first observed by 12 hr postinfection; this was associated with the production of small amounts of intracellular gp160. Mature HIV Gag proteins such as p24 increased between 12 and 24 hr following cocultivation and accumulated intracellularly over the next 24 hr. This program of rapid viral protein synthesis is to be contrasted with the kinetics of HIV protein production observed in the companion cultures infected with cell-free virus; significant levels of p55 Gag were not detected until 72 hr postinfection (see Fig. 1B). The production of progeny virions in the cocultures was similarly accelerated. As shown in Fig. 1C, virus progeny was released into the medium between 16 and 24 hr postinfection, a time frame consistent with the synthesis of HIV proteins 8 to 12 hr following cocultivation. In contrast, the production of progeny virions in the companion cell-free virus infection was not detected until 96 hr.

#### Identification of the DNA polymerase responsible for viral DNA synthesis during cell-to-cell HIV spread

The rapid accumulation of unintegrated HIV viral DNA following cocultivation raised the possibility that these molecules, present but undetectable by Southern blot hybridization in the chronically infected H9 cells, were simply transferred to and amplified in the CD4<sup>+</sup> recipient cells by a reverse transcriptase (RT)-independent mechanism. This possibility was addressed by evaluating the effects of known inhibitors of eukaryotic DNA polymerase- $\alpha$  or reverse transcriptase on the cell-to-cell transmission of HIV. In the first group of experiments to be described, donor and uninfected recipient cells were separately incubated for 1 hr with increasing concentrations of aphidicolin, a known inhibitor of eukaryotic DNA polymerase- $\alpha$  but not bacterial DNA polymerases or reverse transcriptase (Ikegami *et al.*, 1978; Huberman, 1981; Spadari *et al.*, 1982). The treated cells were then cocultivated in the presence of aphidicolin and the production of unintegrated viral DNA was monitored by Southern blotting 4 hr later. As shown in Fig. 2A, the synthesis of HIV viral DNA as a result of cell-to-cell spread was unaffected by concentrations of aphidicolin up to 1  $\mu$ g/ml. As expected, however, cellular DNA replication was completely blocked at an aphidicolin level of 0.5  $\mu$ g/ml.

PFA has been shown to inhibit the RTs associated with avian and mammalian retroviruses including HIV (Sundquist and Lerner, 1977; Sundquist and Öberg, 1979; Sandstrom *et al.*, 1985; Sarin *et al.*, 1985) without significantly perturbing cellular DNA synthesis. As

shown in Fig. 2B, increasing concentrations of PFA had only modest inhibitory effects on [<sup>3</sup>H]thymidine uptake in uninfected H9 cells; however, PFA at a concentration of 60  $\mu$ M completely blocked viral DNA production in the cocultures. Taken together with the aphidicolin experiment described previously, these results imply that reverse transcription and not DNA polymerase- $\alpha$ -dependent DNA synthesis is responsible for the rapid accumulation of viral DNA observed following cocultivation. This conclusion is also supported by the effects of AZT (Mitsuya *et al.*, 1985; Huang *et al.*, 1990) on cell-to-cell virus transmission. As shown in Fig. 2B, the synthesis of HIV viral DNA was markedly inhibited by AZT concentrations of 1.8  $\mu$ M or greater. Both AZT and PFA also blocked virus production in a dose-dependent fashion as measured by RT activity (Fig. 3A).

#### An assessment of potential inhibitors of cell-to-cell HIV infection

Agents known to block cell fusion or specifically suppress the entry of cell-free HIV into susceptible cells were evaluated for their effects on the cell-to-cell spread of virus. Sulfated polysaccharides, such as dextran sulfate and heparin, have been previously reported to block the infection of CD4<sup>+</sup> human T-cells by cell-free preparations of HIV (Ito *et al.*, 1987; Ueno and Kuno, 1987; Nakashima *et al.*, 1987). Although the precise mechanism(s) responsible for this effect is presently unclear, a recent study suggests that these compounds may interact with epitopes on gp120, distinct from CD4 binding sites, and possibly inhibit the fusion of virion and cellular membranes (Schols *et al.*, 1990; Callahan *et al.*, 1991). To evaluate the effect of these agents on the cell-to-cell transmission of HIV, increasing concentrations of dextran sulfate or heparin were preincubated with both donor H9 and uninfected CD4<sup>+</sup> recipient cells for 1 hr prior to mixing; cocultures were then maintained in the presence of similar concentrations of the sulfated polysaccharides for the next 40 hr. Compared to the untreated control cultures, both sulfated polysaccharides markedly suppressed the rapid accumulation of unintegrated viral DNA 4 hr following cocultivation (data not shown) and strongly inhibited virion production as monitored by RT activity at 40 hr (Fig. 3A). In a separate experiment, virus producing and recipient cells were incubated with increasing concentrations of the CD4 monoclonal antibodies Leu3A and OKT4 for 1 hr prior to, as well as 40 hr following, cocultivation; virus production, monitored by RT assay, was strongly inhibited by Leu3A but not by OKT4 (Fig. 3B). Cell-to-cell transmission of HIV could also be blocked by exposing the cocultures to soluble CD4 (Fig. 3B).



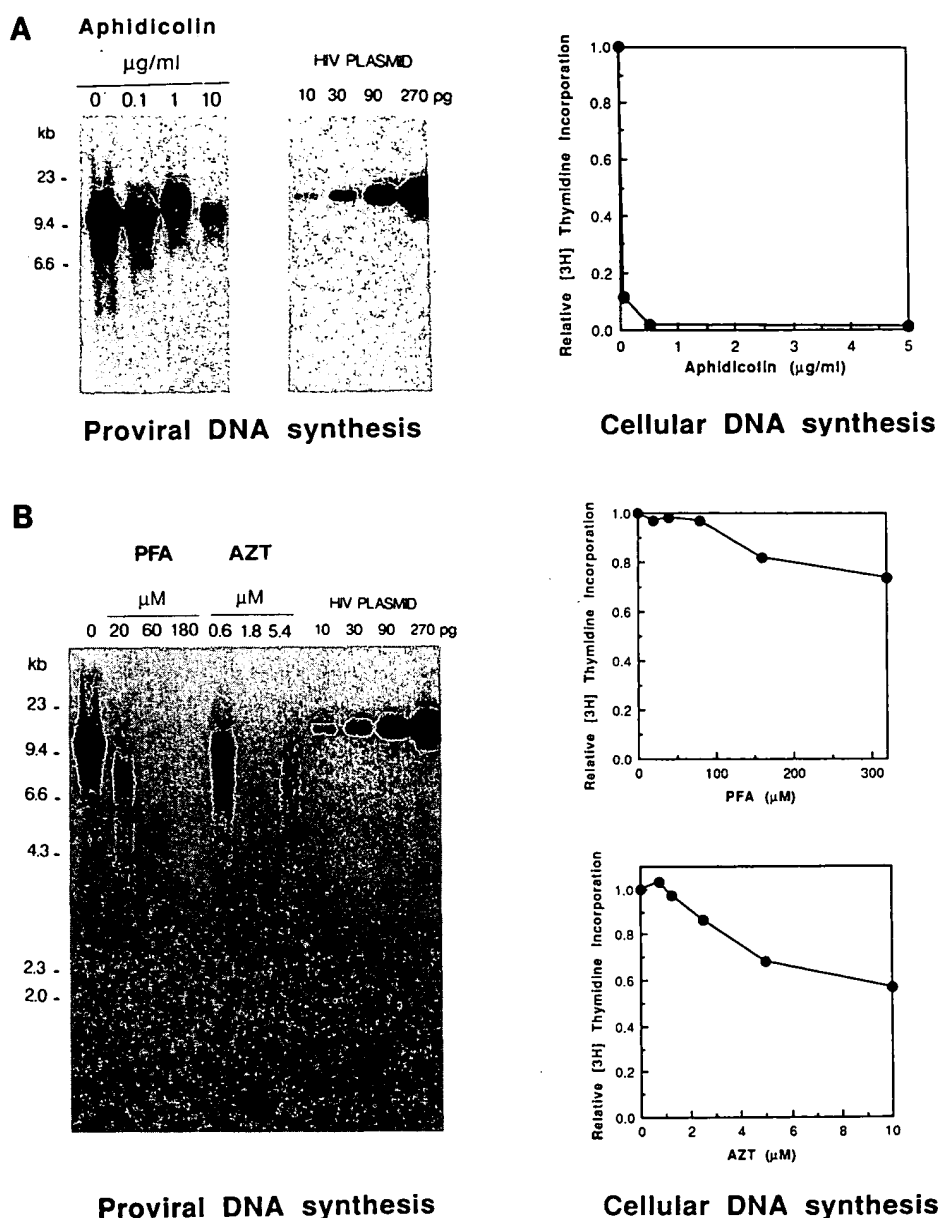


FIG. 2. Identification of the DNA polymerase responsible for viral DNA synthesis during cell-to-cell HIV spread. (A and B, left) Effects of DNA polymerase inhibitors on HIV-1 viral DNA synthesis. H9/HIV<sub>IIIIB</sub> ( $1 \times 10^6$  cells) and uninfected H9 cells ( $4 \times 10^6$  cells) were incubated separately in the presence of the indicated concentrations of inhibitor for 1 hr. The two cultures were then mixed together and incubated for 4 hr in the presence of the same concentration of agent, lysed in Hirt solution, and the Hirt DNAs were subjected to Southern blot analyses. (A and B, right) Effects of DNA polymerase inhibitors on cellular DNA synthesis. Uninfected H9 cells were cultured in the presence of increasing concentrations of inhibitor for 1 hr and then labeled with [ $^3\text{H}$ ]thymidine for an additional 2 hr. Cells were collected and the incorporation of [ $^3\text{H}$ ]thymidine into 5% TCA-insoluble material was determined by liquid scintillation counting. The relative values of incorporated radioactivity in the treated compared to the untreated cultures were calculated from duplicate samples.

#### HIV-induced cell-cell fusion monitored by light and electron microscopy

The experiments presented in Figs. 2 and 3 clearly demonstrate that the extremely rapid HIV replication attending the cocultivation of CD4<sup>+</sup> recipient cells with virus producing H9 cells required: (1) reverse transcrip-

tion and (2) the interaction of gp120 with the CD4 receptor. Although these requirements are also features of HIV infections mediated by cell-free virus preparations, we were intrigued by the seemingly synchronous and reproducibly rapid pattern of viral DNA production in this system. Unintegrated DNA was never detected by Southern blotting during the first 2 hr of cocultiva-

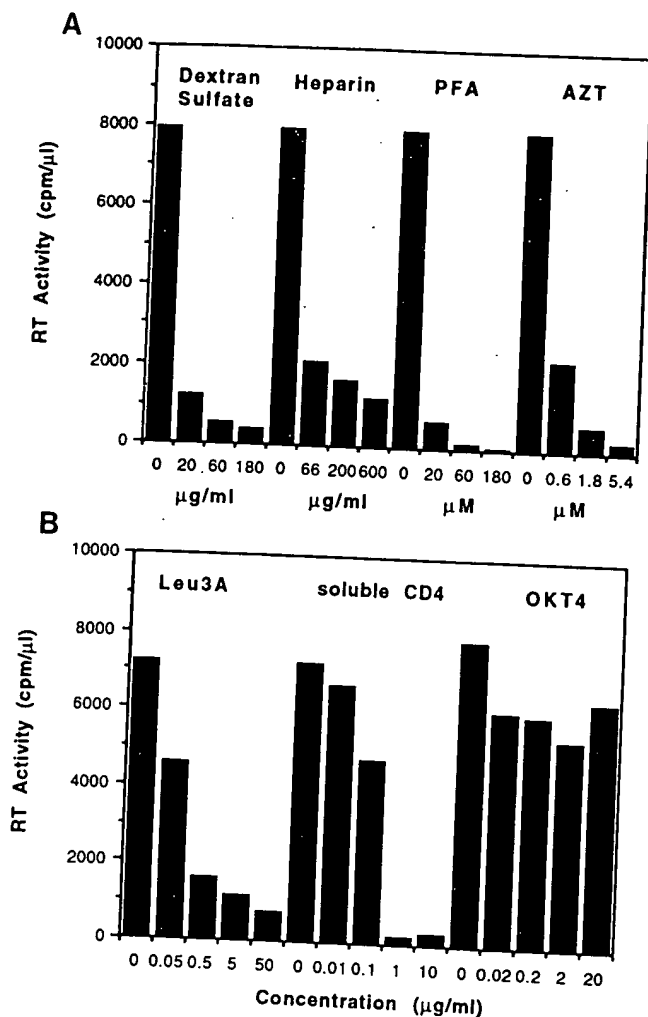


Fig. 3. Effects of sulfated polysaccharides (A), DNA synthesis inhibitors (A), monoclonal antibodies (B), and SCD4 (B) on progeny virion production during cell-to-cell spread of HIV. H9/HIV<sub>III</sub> ( $2 \times 10^5$  cells/ml) and H9 cells ( $8 \times 10^5$  cells/ml) were separately cultured in the presence of the indicated concentrations of inhibitor for 1 hr, mixed, and cultured for an additional 40 hr in the presence of the same concentration of agent. Culture supernatants were collected and RT activity was measured using [ $^{32}$ P]TTP.

tion but by 4 hr, a burst of DNA synthesis resulted in the accumulation of at least 40 DNA copies per cell. Because of the apparent similarity to cell-free virus infections, we favored "high local concentrations" of virus particles on the surface of the donor cells as the most likely initiator of virus replication in the cocultures since they would utilize CD4 for entry and require RT for the production of unintegrated DNA.

Since the critical steps of cell-to-cell virus transmission occurred during the first 4 hr of cocultivation, we decided to initially monitor this early phase of infection by light and electron microscopy. Individual cocultures were fixed in 2.5% glutaraldehyde at 30 and 60 min

and at 2, 4, 8, and 16 hr following cocultivation as described in Fig. 4. As controls, the chronically infected H9 cells were incubated on ice for various periods of time with or without H9 cells or at 37° with a fourfold excess of CD4-negative A2.01 cells. Low-power light microscopy revealed unambiguous multinucleated cells by 1 hr of cocultivation; over the next several hours, the number and size of these cells increased dramatically (Fig. 4). At 1 hour, the multinucleated cells contained as many as 10 nuclei in a single plane of section; the number of nuclei increased to 20 by 2 hr and to 40 by 4 hr. In the larger giant cells present at 8 and 16 hr, the nuclei formed "rings" around central clear Golgi zones. Degenerating multinucleated cells were first detectable at 4 hr, and by 8 hr nuclear pyknosis and karyorrhexis and cytoplasmic ballooning/blebbing were evident. In contrast, the cocultures of the virus producing H9 cells/A2.01 cells (Fig. 4G) and the cocultures maintained on ice exhibited no fusion, multinucleation, or cell degeneration/necrosis.

Fusion associated with cell-to-cell virus transmission could be detected within 15 min of cocultivation by electron microscopy (Fig. 5). Progressively enlarging areas of aligned plasma membranes of adjacent cells were regularly observed. Within such zones, cell fusion was observed either at one or more sites ("skip" areas) or involved the entire area visible in the plane of section and exhibited a loss of the lipid bilayers as well as blending of cytoplasm (Figs. 5A and 5B). An unexpected feature of this process was the apparent lack of budding or mature virus particles in association with the fusing plasma membranes (Fig. 5C). The virions on the surface of fusing cells had the same random distribution as that seen in the nonfusing H9<sub>III</sub>/A2.01 cocultures. When virions were present, alignment and fusion appeared to proceed around them as if the particles were trapped or even an impediment to fusion (Fig. 5D). Virions were rarely observed associated with the very smallest areas of membrane alignment or fusion where they could be expected to be visible in the plane of section.

#### HIV-induced cell-cell fusion monitored by fluorescent dye redistribution and sCD4 inhibition

The rapidity of the HIV-induced cell-cell fusion could also be demonstrated by two additional independent methods. In the fluorescent dye redistribution experiment shown in Fig. 6A, chronically infected H9 cells, containing the water-soluble BCECF-AM dye, were mixed with a 100-fold excess of uninfected H9 or CD4-negative A2.01 cells. No evidence of fusion was evident following 10 min of incubation; by 15 min,

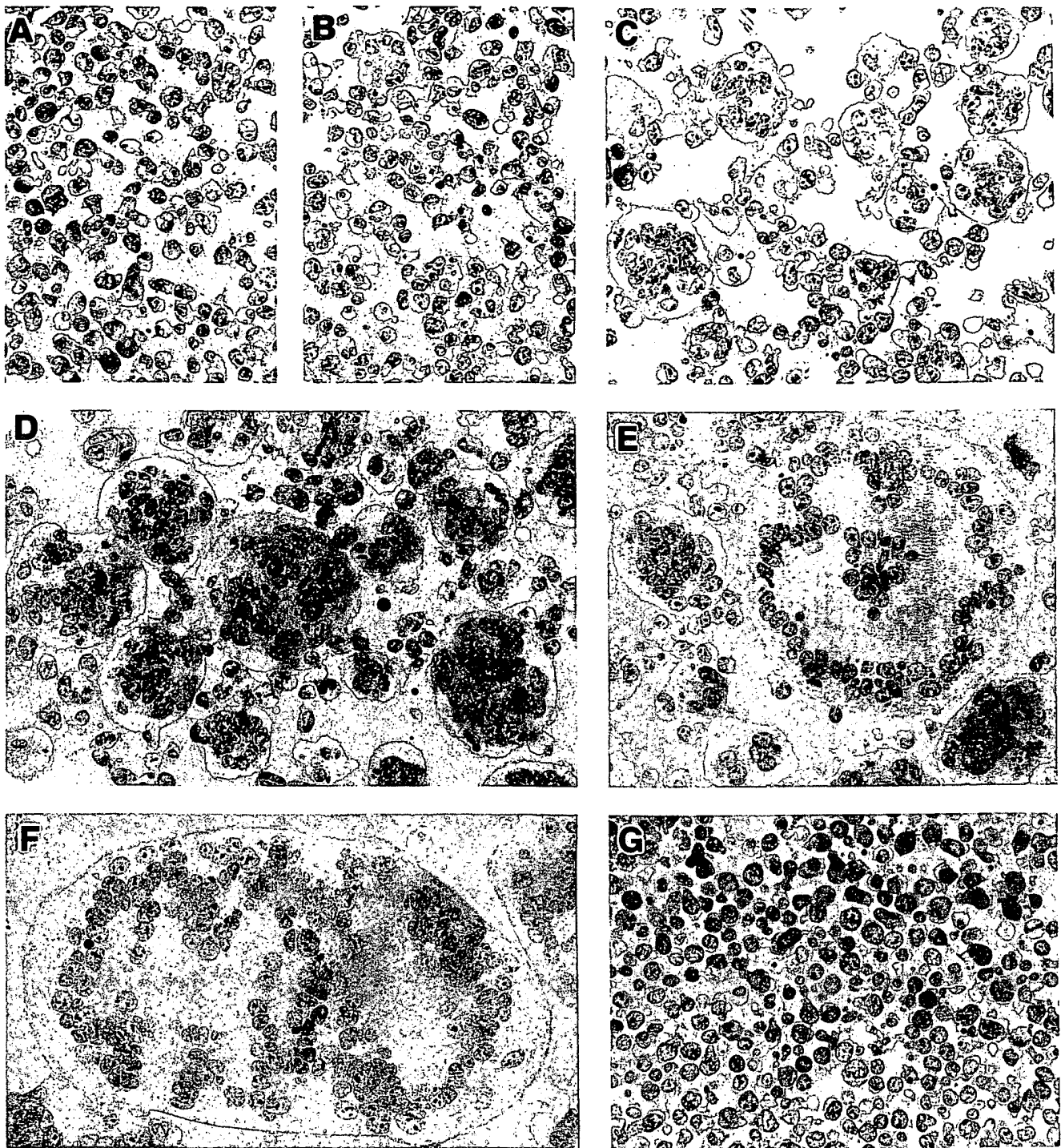
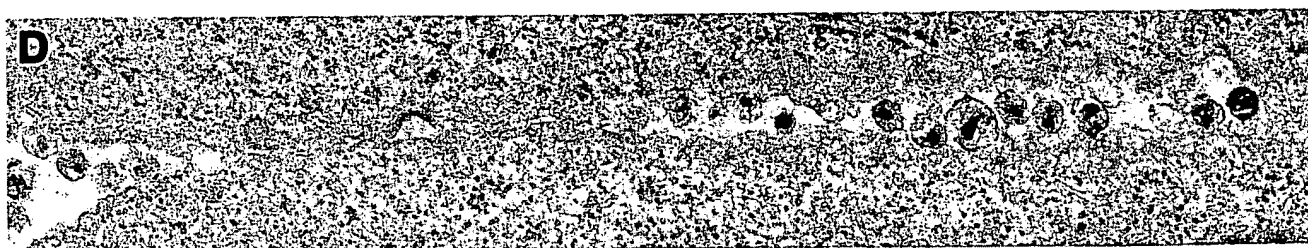
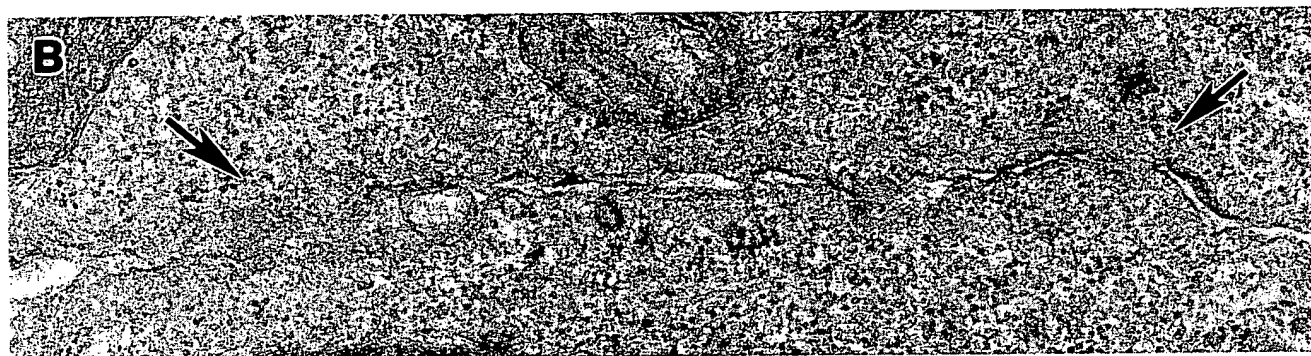
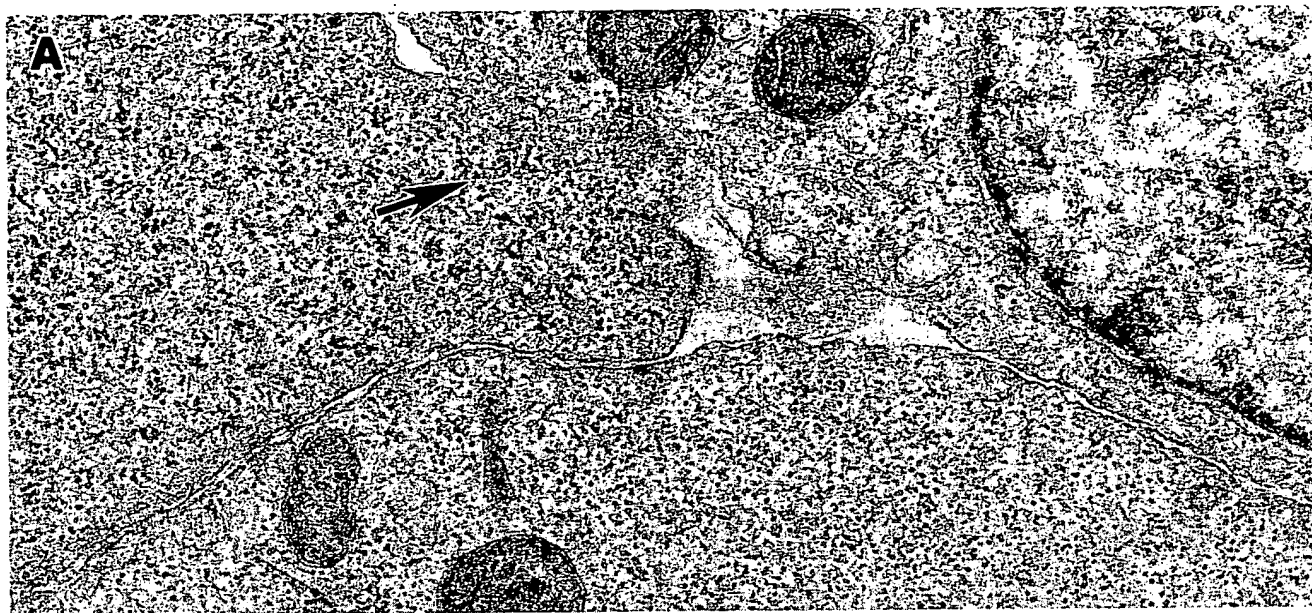


FIG. 4. Cell-cell fusion monitored by low-power light microscopy. Individual H9/HIV<sub>III</sub>-H9 cocultures were established as described under Materials and Methods. At the indicated times, cells were fixed in 2.5% glutaraldehyde, pelleted in a microfuge, gelled into cold agar, dehydrated, and embedded in Spurr's plastic. H9/HIV<sub>III</sub> cells were also cocultured with A2.01 cells, a CD4-negative, clonal derivative of CEM cells, at a ratio of 1:4 for 4 hr (4 hr + A2.01). One-micrometer plastic sections were stained with toluidine blue. Magnification:  $\times 285$ . (A) 30 min; (B) 60 min; (C) 2 hr; (D) 4 hr; (E) 8 hr; (F) 16 hr; (G) 4 hr + A2.01.

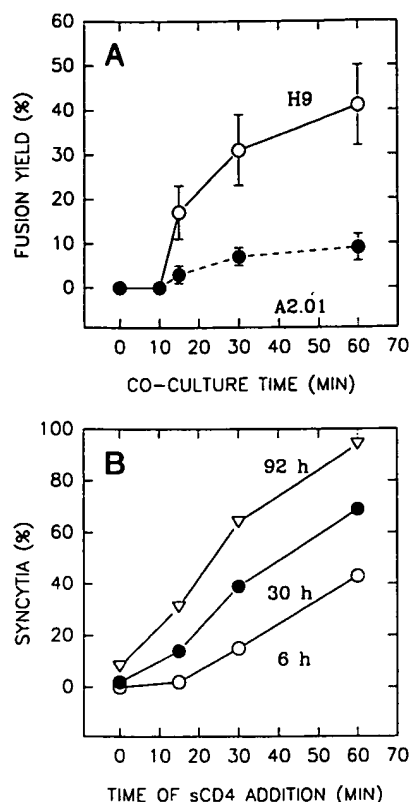


doublets or higher order cell aggregates containing fluorescent dye were readily detected in the cocultures with H9 cells compared to the control A2.01 cells. In a separate experiment, the blocking effect of high concentrations of soluble CD4 on syncytia formation was monitored in cocultures containing H9/HIV<sub>III</sub>B and uninfected H9 cells mixed at a ratio of 1:10. As shown in Fig. 6B, syncytia were apparent even when blocking concentrations of sCD4 were added as soon as 15 min following cocultivation.

## DISCUSSION

The ability to induce cell fusion is a property shared by a diverse group of enveloped animal viruses. This aspect of the virus life cycle has been intensively studied in several different paramyxovirus systems where cell-to-cell spread may accompany infection initiated by released virus particles. Of particular relevance to the work reported here are paramyxovirus systems in which extracellular particles are not required for cell-to-cell virus spread (Merz *et al.*, 1980). In the case of paramyxovirus simian virus 5 (SV5), monospecific antibodies directed against glycoproteins which mediate virus adsorption (HN glycoprotein) or penetration/fusion (F glycoprotein) neutralize cell-free virus infections if incubated with virus *prior* to absorption. However, when virus producing cells are treated with the two antibody preparations 9 hr postinfection, only the antibody specific for the F protein blocks cell-to-cell virus spread, effectively confining the infection to the initial focus of SV5 replication. In the cells exposed to HN antibody, progeny virions are produced and released into the medium but are unable to adsorb to the uninfected cells. Nonetheless, multinucleated cells, the hallmark of cell-to-cell virus transmission, soon appear in the cultures incubated with HN antibody. Thus the cell-to-cell spread of SV5 simply requires expression of the F protein but not the production or release of progeny virions.

Our electron microscopic examination of the early stages of cell-to-cell HIV transmission strongly suggests that the cell-cell fusion and initiation of unintegrated viral DNA synthesis do not involve the participation of nascent or formed viral particles generated by



**FIG. 6.** Cell-cell fusion monitored by fluorescent dye redistribution and sCD4 competition. (A) Chronically HIV-infected H9 cells were labeled with BCECF-AM and mixed with uninfected H9 or CD4-negative A2.01 cells at the ratio of 1:100. Samples were removed at the indicated times and examined by fluorescent microscopy. Fluorescent cells (individual as well as higher order aggregates) were counted in three different fields of duplicate wells. The fusion yield was defined as the number of fluorescent cells present in aggregates (usually doublets and triplets) divided by the total number of labeled cells multiplied by 100. (B) Uninfected H9 cells were mixed with H9/HIV<sub>III</sub>B cells, and sCD4 was added to the media at the indicated times following cocultivation. The number of fused cells was counted by light microscopy following 6, 30, and 42 hr of cocultivation. The fusion yield of each culture was defined as the number of syncytia formed in the presence of sCD4 divided by the number of syncytia present in its absence (96 and 110 syncytia detected at 6 and 92 hr, respectively) multiplied by 100.

the donor cell. It is impossible to totally eliminate, by transmission electron microscopy, the possibility that cell-cell fusion requires the participation of virions.

**Fig. 5.** Electron microscopy of HIV cell-to-cell transmission. Cells were cocultivated, fixed, and processed into plastic as described under Materials and Methods. Thin sections were stained with uranyl acetate and lead citrate and examined on a Zeiss EM10A electron microscope. (A) Portions of the plasma membranes of three H9 cells are closely aligned; fusion has begun in one area with commencement of cytoplasmic mixing (arrow). No budding or mature virions are in the vicinity. Fifteen minutes of incubation;  $\times 39,000$ . (B) Fusion has begun at several locations (arrows) along the aligned plasma membranes. No virions are observed. Fifteen minutes of incubation;  $\times 49,000$ . (C) Typical budding and mature HIV particles are near, but apparently not associated with the aligned plasma membranes of two cells. Thirty minutes of incubation;  $\times 24,000$ . (D) Free mature virions appear trapped between areas of fusing plasma membranes. It is even unusual to see this many virions so located. Sixty minutes of incubation;  $\times 15,400$ .



However, not only were virus particles and budding structures relatively rare in the areas of fusion (both "skips" and extended aligned regions), but they were not visualized in even the smallest zones of fusion where they would have been expected to be detected in the plane of section. In this regard, the cell-cell fusion mediated by Sendai virus particles has been investigated at 28° rather than 37° to slow down the fusion process (Kim and Okada, 1981; Okada, 1987). We have carried out an experiment under similar conditions (donor and recipient cells were separately incubated at on ice for 15 min, mixed, and then incubated at 28° for various times up to 30 min) and again observed rapid cell fusion; no HIV particles were observed participating in the fusion process (data not shown).

Our inability to detect virion structures associated with plasma membrane fusion is compatible with published results demonstrating that cells, infected with vaccinia virus recombinants expressing only HIV envelope proteins (gp160 coding sequences), are able to form syncytia following cocultivation with CD4<sup>+</sup> cells (Lifson *et al.*, 1986). In the latter system in which no HIV particles are produced, the surface expression of gp120/gp41 directs cell fusion. It is very likely that HIV-infected cells also become "coated" with gp120/gp41 complexes, a majority of which may not be associated with viral core structures. In the cell-to-cell transmission of HIV we have described, these cell surface envelope glycoproteins undoubtedly drive the critical fusion step which is required to transfer the infection to adjacent cells. Since reverse transcription is particle-associated in all known retrotransposon/retrovirus systems and an obligatory reverse transcription step is required for efficient cell-to-cell spread of HIV (Fig. 2), it must be assumed that some type of viral ribonucleoprotein complex, perhaps a structure identifiable only by immune electron microscopy, carries out this important function.

The experiments described above provide little information regarding the mechanism(s) underlying the induction of high levels of HIV replication following cell fusion. In this regard, the activation of virus replication in the *donor* rather than the recipient cells as a consequence of the fusion event cannot be formally ruled out. For example, alterations in plasma membrane structure might affect intracellular pH or ion concentrations while the introduction of additional cytoplasm or nuclei might perturb cytoskeleton elements thereby stimulating vigorous virus replication. What is clear is that the mixing of donor and recipient cell cytoplasm following fusion provides a fertile environment for efficient virion production. It is possible that the recipient cytoplasm supplies a factor depleted from the donor

cell or inactivates an inhibitor present in the donor cell. Experiments are currently in progress assessing each of these issues.

There are several implications of this work that impact on current thinking about HIV infections *in vivo*. In the side-by-side comparison of virus infections initiated by infected cells or cell-free virus derived from the same cells, the former was a far superior inoculum as monitored by viral DNA, protein, and progeny virion production (Fig. 1). Assuming that a seronegative individual is initially exposed to HIV as a mixture of infected cells and cell-free virus and the relative amounts of each are similar to that present in our cocultivation system, then cell-to-cell transmission would represent the more efficient and relevant mode of initiating *de novo* infection. Furthermore, it is very likely that cell-to-cell virus spread sustains the persistent and asymptomatic HIV infection lasting many months to years, during which time plasma viremia may be difficult to demonstrate.

It should be stressed that the system we have developed to monitor virus-induced cell-cell fusion should be viewed as a model and *not* a replica of events occurring in an HIV-infected individual. It has been reported that virus isolated from seropositive individuals usually replicates slowly to only low titers and is unable to infect continuous human T-cell lines (Åsjö *et al.*, 1986; Evans *et al.*, 1987). Another common property of these virus isolates is their failure to induce ballooning syncytia as monitored by inverted microscopy (Cheng-Mayer *et al.*, 1988; Tersmette *et al.*, 1988; Fenyo *et al.*, 1988). Thus it is very unlikely that cell-cell fusion, involving *circulating* mononuclear cells, occurs *in vivo* in view of the low concentrations of virus-infected cells coupled with hydrodynamic factors which minimize prolonged cell-to-cell contact. In contrast, HIV-induced cell-cell fusion would be favored in lymph nodes and spleen in view of the high concentrations [ $10^7$ – $10^9$  cells/ml (Layne *et al.*, 1991)] of "slowly moving" lymphocytes reported to be present in these lymphoid tissues.

Finally, the rapidity and efficiency of the cell-cell fusion associated with cell-to-cell HIV transmission represents a potential formidable impediment to vaccine development. Assuming that the targets of a protective immunological response are cells rather than virions, then the immune system must be primed to deal with and eliminate such inocula in a matter of minutes to hours. By analogy to paramyxovirus systems, an effective HIV vaccine must be directed against cell fusion as well as infection mediated by virus particles. Current strategies of vaccine development primarily focus on gp120 and are directed against domains such as the V3 loop which is thought to participate in the fusion event. Equally plausible are strategies that target all or

parts of gp41 in order to prevent the dissemination of virus as a consequence of cell-cell fusion.

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ulations must be considered. We totally agree with his arguments. His constructive, detailed technical explanations should be tested thoroughly by the manufacturers of Ag/Cu ionization systems and taken into consideration in future experiments.

Dr. Lin's main scientific point is that the data we presented in our paper do not support the claim that *Legionella* developed resistance to silver. He assumes that Ag/Cu ionization in our hospital did not effectively control *Legionella*, even in the beginning. To support this, he gives the percentages of water samples from distal sites that were positive for *Legionella* (detection limit 1 cfu/L). However, we cannot draw valid conclusions from these values alone. Our statistical evaluation is based on *Legionella* counts (cfu/L) and not on "sample points positive for *Legionella*." The results of the multiple regression analysis that we presented in our paper clearly revealed a decreased influence of Ag ions on *Legionella* counts during the 4-year study period. But even without performing a statistical evaluation the facts are as follows: in the first year after the Ag/Cu ionization unit was installed, the percentage of samples positive for *Legionella* decreased from 100% to 55%, with an average Ag level of <10 µg/L; in the fourth year of Ag/Cu ionization, the percentage of samples positive remained at 75%, with an average Ag level of 30 µg/L.

The methods and detection limits used in various reports concerning Ag/Cu ionization are not comparable. In order to facilitate comparison [2], we gave results as counts of *Legionella* cfu/L, not as positive distal sites per swab [3, 4]. We prefer a quantitative method in reporting effective disinfection, which is a common procedure in examining any disinfection method (see, e.g., [5]). Another question is whether there is any connection between the quantity of *Legionella* (cfu/L) in water distribution systems and the incidence of legionnaires disease. Indeed, there are many unresolved questions regarding the effectiveness of Ag/Cu ionization for control of *Legionella* in hospitals, including the influence of chemical water composition, temperature, and circulation on metal activity.

Vigilance is necessary not only because *Legionella* may develop resistance to the activity of Ag and Cu, but also because of the question of protozoa inactivation by Ag and Cu ions. We believe that methods for disinfecting water distribution systems should be designed to control the growth of both *Legionella* and protozoa. But the investigations of Cassels et al. [6] showed that electrolytically-generated concentrations up to 80 µg/L Ag and 800 µg/L Cu did not inactivate *Naegleria fowleri* in vitro. Recently we reported that *Hartmannella vermiformis* survived at concentrations of 50 µg/L Ag and 500 µg/L Cu in vitro [7].

There was further discussion of the arguments about Ag/Cu ionization at a panel discussion on "Copper/Silver Water Ionization Systems: Pro and Con" at the Fifth International Conference on *Legionella* at Ulm, Germany, 26–29 September, 2000.

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#### Limitations of Plasma Human Immunodeficiency Virus RNA Testing

**STR**—The studies of Mezzaroma [1] illustrate the limitations of plasma HIV RNA testing and the need for additional standardized assays to measure viral dynamics in HIV-infected patients. In a recent study [2] we compared the CD4<sup>+</sup> and CD8<sup>+</sup> cell counts and the levels of HIV DNA, HIV RNA, and infectious HIV in patients who partially responded to highly active antiretroviral therapy (HAART) and in patients for whom HAART failed completely. Patients who responded to HAART had increasing levels of CD4<sup>+</sup> cells, and patients who did not had decreasing levels of CD4<sup>+</sup> cells. Although plasma HIV RNA levels were similarly high in both groups, when compared with patients who did not respond to HAART, the patients who did respond had significant increases in CD8<sup>+</sup> cells, fewer positive plasma HIV cultures, lower frequencies of infectious HIV in CD4<sup>+</sup> cells, and lower frequencies of HIV DNA in

CD4<sup>+</sup> cells. These studies suggest that measuring the levels of infectious HIV and HIV DNA may be of value to individuals with discordant immunological and virological responses to HAART. Replication of HIV continues even in individuals whose plasma HIV RNA levels have become undetectable as a result of HAART [3], and these patients may also benefit from tests that measure the cellular reservoir of HIV and/or HIV production.

Although measurement of plasma HIV RNA is currently the gold standard for predicting the likelihood of HIV disease progression, it may only be showing us only the tip of the iceberg. Microculture titrations of infectious virus provide information about the levels of replication-competent virus, but the cost of these assays makes it unlikely that they will become available for routine clinical use. The findings that patients without disease progression over many years have lower levels of HIV DNA in peripheral blood cells than do patients with rapid progression, and that the levels of cellular HIV DNA decreases after initiation of HAART, suggest that this parameter may be a useful indicator of HIV disease progression [4]. Measurement of circular forms of HIV DNA as an index of ongoing HIV replication may also prove to be useful for the clinical management of HIV-infected patients [3]. Given the apparent stability of the proviral HIV DNA levels, analysis of this marker (i.e. total HIV DNA minus the circular forms of HIV DNA) may be a useful marker of vaccine efficacy, while measurement of RNA levels will only be useful in the very short period of time between the identification of infection and the initiation of HAART. Recent studies [5] suggest that measurement of levels of peripheral blood cells expressing the gp120 envelope protein of HIV might also be useful for monitoring HIV-infected individuals. Longitudinal studies that compare the cellular reservoir of HIV, plasma HIV RNA, and immunological

parameters are urgently needed to determine the best strategies for the management of HIV disease and to elucidate the viral and immunological dynamics that determine clinical end points in HIV infection.

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## Surface CD4 density remains constant on lymphocytes of HIV-infected patients in the progression of disease

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### SUMMARY

In an attempt to question the influence of circulating virus, soluble gp120 or CD4 self-reacting antibodies upon results of CD4<sup>+</sup> T-cell immunophenotyping in AIDS patients, five anti-CD4 mAb defining several epitopes of the V1 and V2 domains of the CD4 molecule were used to analyse the epitopic density of CD4 on lymphocytes of seropositive patients taken at stages II, III and IV of HIV infection, according to the Centers for Disease Control (CDC, Atlanta) classification. Our results demonstrate that each CD4 epitopic density measured on circulating lymphocytes remains constant at a mean level of 46,000 epitopes per cell whatever the stage of the disease and whatever the serum p25 concentration. These data provide evidence that antibody accessibility to several CD4 epitopes is not altered by putative interactions between CD4 molecules and circulating virus, soluble gp120 or anti-CD4 autoantibodies. If such binding events, as expected, do occur *in vivo*, they are of too low a magnitude to influence the immunophenotyping. Furthermore, we show that mAb specific for different epitopes in the V1 and V2 domains of the CD4 molecule can be used interchangeably for the biological followup of the CD4<sup>+</sup> cell population in blood samples of HIV-infected patients.

**Key-words:** T lymphocyte, CD4, AIDS, HIV; Phenotyping, V1-V2 domains.

### INTRODUCTION

The number of helper-inducer (CD4<sup>+</sup>) lymphocytes represents an important characteristic in the classification, prognosis and response to

treatment of persons infected with the human immunodeficiency virus (HIV) (Zolla-Pazner *et al.*, 1987) and its measurement has been generalized in the last 3 years. However, little is known about potential fluctuations in the cell surface

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expression level of the CD4 molecule itself and/or of its various epitopes during the evolution of AIDS disease.

Such fluctuations, the evaluation of which may be of prognostic interest, could, for instance, be due to site-related hindrance mediated by circulating virions, soluble gp120 envelope proteins (Weinhold *et al.*, 1988), CD4 self-reacting antibodies (Kowalski *et al.*, 1989) or CD4 modulation (Hoxie *et al.*, 1986; Salmon *et al.*, 1988), phenomena which could, in part, account for immunodeficiency.

In theory, various patterns of CD4 epitope expression might be observed in HIV-infected patients as a result of different physiopathological situations, including the following, which are investigated here: first, HIV-mediated helper T-cell death or complete CD4 downregulation, leaving intact a number of lymphocytes with normal cell surface expression of CD4 molecules; and second, binding of viral gp120 or CD4-self reacting antibodies to CD4 molecules, either inducing the internalization of some CD4 molecules resulting in lymphocytes with low cell surface expression of CD4 molecules, or masking some, but not all, CD4 epitopes, resulting in lymphocytes expressing different epitopic CD4 densities.

To distinguish between these 3 hypotheses, we performed cytofluorometric quantification of 5 different CD4 epitopes on lymphocytes of HIV-infected patients at different stages of the illness.

## PATIENTS AND METHODS

### Clinical samples

Blood samples from "normal" HIV<sup>-</sup> subjects and HIV-infected patients selected from a cohort of volunteers followed at the Conception Hospital (CHU, Marseille, France) and including persons at various stages of the disease (stage II to IV), according to the Centers for Disease Control (CDC Atlan-

ta classification) were recovered in heparinized tubes (Vacutainer, Becton-Dickinson, Mountain View, CA) for immunophenotyping (see below). P25 antigenaemia in serum samples was evaluated using a commercial kit (HIV-EIA, Abbott Lab., Wiesbaden, Germany) following manufacturer's instructions.

### Cell lines

The HTLV-I-immortalized CD4<sup>+</sup> T-cell line MT4, highly susceptible to the cytopathogenic effect of HIV1, originally derived from an acute T leukaemia patient, was kindly provided by N. Yamamoto (Harada *et al.*, 1985). HIV-highly susceptible subclone 3 was derived from the CD4<sup>+</sup> lymphoblastoid CEM cell line, mycoplasma-free, purchased from the American Type Culture Collection (ATCC). Cells were cultivated in RPMI-1640 supplemented with 1 % PSN antibiotic mixture, 1 % glutamine (Gibco, Paisley, Scotland, UK) and 10 % foetal calf serum (Biological, Jerusalem, Israël).

### Monoclonal antibodies

The following anti-CD4 monoclonal antibodies (mAb) were used: OKT4A (IgG<sub>2a</sub>) and OKT4 (IgG<sub>2b</sub>), purchased from Ortho (Ortho Diagnostic Systems, Inc., Raritan, NJ, USA), IOT4/BL4 (IgG<sub>1</sub>) and IOT4A/13B8-2 (IgG<sub>1</sub>) provided by Immunotech (Immunotech SA, Marseille, France). ST4/F.101.69 (IgG<sub>1</sub>), ST40/F142.63 (IgG<sub>1</sub>) and the cocktail ST440 (ST4 + ST40) obtained from Biosys (Biosys, Compiègne, France), anti-Leu 3a + 3b cocktail purchased from Becton-Dickinson (Becton-Dickinson, Mountain View, CA, USA).

The following control mAb were used: anti-CD2 ST11 (IgG<sub>1</sub>), anti-CD8 ST8 (IgG<sub>1</sub>) and anti-CD45 SLC1 (IgG<sub>1</sub>) provided by Biosys, anti-CD8 B9-11 (IgG<sub>1</sub>) and anti-CD14 IOM2 (IgG<sub>1</sub>) obtained from Immunotech.

### mAb competitive inhibition assay

The spatial relationships between epitopes detected by the various mAb were studied by competitive binding inhibition of <sup>125</sup>I-iodine-labelled mAb to CEM cells using increasing amounts (10<sup>-4</sup> to 10<sup>4</sup> ng) of unlabelled competitor mAb as previously described (Pierres *et al.*, 1981).

AIDS	= acquired immune deficiency syndrome.
CDC	= Centers for Disease Control.
cpm	= counts per minute.
HIV	= human immunodeficiency virus.

mAb	= monoclonal antibody.
MFI	= mean fluorescence intensity.
PBS	= phosphate-buffered saline.
TCID <sub>50</sub>	= 50 % tissue culture infective dose.

## HIV infection assay

Either  $5 \times 10^5$  CEM or  $3 \times 10^5$  MT4 were incubated for 1 h at  $4^\circ\text{C}$  in a 96-well flat-bottomed microwell plate (Costar, Cambridge, MA, USA) with saturating amount of mAb, as determined by competitive inhibition assay, into 100  $\mu\text{l}$  of culture medium. Cells were exposed for 1 h at  $4^\circ\text{C}$  to 100  $\mu\text{l}$  HIV1(HIV1-BRU, the HIV1 prototype strain)-chronically-infected CEM supernatant (reverse transcriptase activity  $1.5 \times 10^3$  cpm/ml)  $10^3$  diluted corresponding to 100 50 % tissue-culture-infective-doses (TCID<sub>50</sub>). Cells were washed 5 times and then cultivated at  $37^\circ\text{C}$  in a 5 %  $\text{CO}_2$  atmosphere in duplicate in 1-ml culture medium at  $3 \times 10^5$  MT4 or  $5 \times 10^5$  CEM per ml in 24-microwell plate (Costar). Infection of the MT4 cells by HIV1 was followed daily by scoring the cytopathic effect (syncytia formation and cell death) under an optical microscope (Rey *et al.*, 1987). Infection of CEM cells was monitored twice a week by measuring reverse transcriptase activity in the cell-free supernatant as previously described (Rey *et al.*, 1984).

## Immunophenotyping in quantitative flow cytometry

Cell staining was performed by indirect immunofluorescence on whole blood using an adaptation of the method of Hoffman *et al.* (1980). Each blood sample was distributed (100  $\mu\text{l}$ ) into 1-ml tubes (Scatron, Osi, France), each containing the appropriate anti-CD4 or control mAb (100  $\mu\text{l}$ ) at saturating concentration (2, 5, 10, 15 and 100  $\mu\text{g}/\text{ml}$  for ST4, OKT4A, 13B8.2, ST40 and BL4, respectively). After 1-h incubation at  $4^\circ\text{C}$  and 2 washes, 50  $\mu\text{l}$  of a saturating amount of sheep anti-mouse IgG (Fab')<sub>2</sub> FITC-labelled 1/25 diluted (Silenus, Eurobio, Paris) was added on pelleted cells and incubated for 30 min. Red cells were lysed for 10 min at  $37^\circ\text{C}$  with  $\text{NH}_4\text{Cl}$  lysing solution.

After 3 additional washes with PBS/0.1 %  $\text{NaN}_3$ , pellets were fixed in 600  $\mu\text{l}$  paraformaldehyde 1 % in PBS for one night at  $4^\circ\text{C}$ , washed and stored in PBS/0.1 %  $\text{NaN}_3$  until analysis.

Controls included the CD4 cocktails anti-leu 3a+3b and ST440, ST8 and ST11 for enumeration of  $\text{CD4}^+$ ,  $\text{CD8}^+$  and total T lymphocytes, respectively and IOM2 and SLC1 to check lymphocyte contamination by monocytes and red cell debris.

Fixed samples were analysed using an "Epics Profile" (Coultronics, Margency, France). Lymphocytes were gated on the basis of dual scatter, and green fluorescence was simultaneously recorded in linear amplification for quantification and log amplification for easier enumeration of positive cells. Mean fluorescence intensity (MFI) was recorded in the positive cell region, corrected from background (nega-

tive control MFI) and plotted on a standard curve relating the net MFI measured in flow cytometry with the mean number of mAb molecules bound per cell. The standard curve was constructed as previously described (Poncelet and Carayon, 1985; Poncelet *et al.*, 1986) by using five stable CCRF-CEM subclones (CEM0.5, CEM5, CEM25, CEM44 and CEM99) expressing 500, 5,000, 25,000, 44,000 and 99,000 CD5 molecules per cell, respectively, as biological standards of known mAb (T101 anti-CD5 mAb) density. This technique enabled us to monitor cell surface CD4 disappearance in the course of *in vitro* HIV infection of a CEM cell line (fig. 1).

## RESULTS

## CD4 epitope mapping

We first defined the spatial relationships between the 5 epitopes identified by the anti-CD4 mAb selected for the immunophenotyping study. Epitope specificities of these 5 mAb, OKT4A, 13B8-2, BL4, ST4 and ST40, were compared in

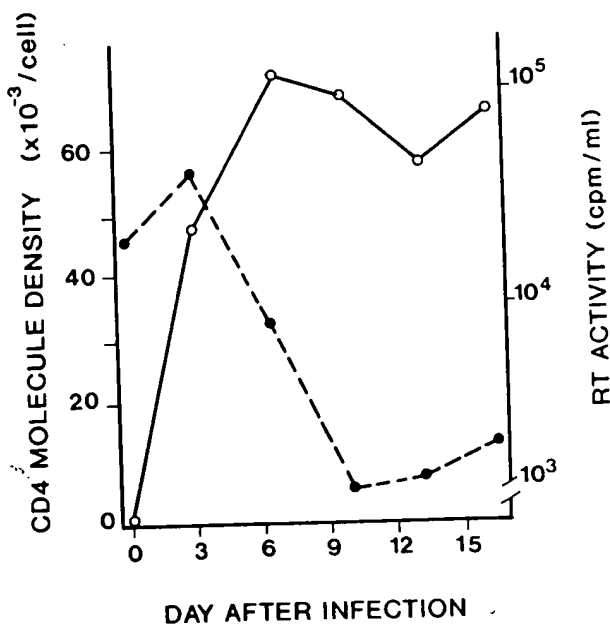


Fig. 1. Cell surface CD4 density in the course of *in vitro* HIV infection.

CEM cells were infected by HIV1 and 13B8-2 epitope cell surface concentration ( $\bullet$ ) and culture supernatant RT activity ( $\circ$ ) was measured twice a week as described in "Patients and Methods".

a binding competition assay (table I). Symmetric competition was observed between ST4 and OKT4A, 13B8-2 and ST4, and ST40 and 13B8-2, whereas BL4 and OKT4 apparently recognize different epitopes apart from the others. This extends the observations of Sattentau *et al.* (1986) and correlates with the previously described mapping by directed mutagenesis (Peterson and Seed, 1988; Mizukami *et al.*, 1988; Clayton *et al.*, 1988; Sattentau *et al.*, 1989) linking OKT4A to amino-acid position 57-67, 13B8-2 to position 87-89, ST4 to position 42-43 in V1, BL4 to position 94-122 in V2 and OKT4 to position 155-158 in the V3 domain of CD4, with gp120 being linked to position 42-49 and 52-57.

Next we determined, among these 5 epitopes, those spatially related to the HIV-binding site on CD4. We thus studied the capacity of the different anti-CD4 mAb to interfere with gp120-CD4 binding by analysing the HIV infection of MT4 and CEM cells pre-incubated with the mAb before viral exposure. Results in table I show that only OKT4A was able to protect cells from HIV infection.

### Lymphocyte CD4 cell surface density in the course of AIDS

Patient groups included HIV<sup>-</sup> controls (n=5), HIV<sup>+</sup> phase II (n=11), HIV<sup>+</sup> phase III (n=26) and HIV<sup>+</sup> phase IV (n=17); p25 antigenaemia was determined for all patients. Four patients in stage II and 9 patients in stage IV had positive p25 antigenaemia.

For each anti-CD4 mAb, the mean number of bound mAb molecules per cell was calculated from the MFI as described in "Patients and Methods", and defines the epitopic density. Figure 2A shows the distribution among patient groups of the whole CD4 molecule density, calculated as the mean of the five epitopic densities. As can be observed and confirmed by statistical analysis, the whole CD4 molecule density remains constant at a level of  $46,000 \pm 6,000$  molecules per lymphocyte whatever the phase of the disease and the serum p25 concentration.

Figure 2B shows the distribution of the 5 different CD4 epitopic densities among patient groups. The density of each CD4 epitope is constant between patient groups. Yet, for any given

Table I. Summary of CD4 epitope mapping by mAb cross-blocking analysis and HIV1 infection inhibition.

Competitor mAb	Binding of <sup>125</sup> I-labelled mAb (a)					Infection of CD4 <sup>+</sup> cells with HIV1 BRU (b)
	OKT4A	ST4	13B8.2	ST40	BL4	
OKT4A	—	—	++	++	++	—
ST4	—	—	—	++	++	++
13B8.2	++	—	—	—	++	++
ST40	++	++	—	—	++	++
BL4	++	++	++	++	—	++
OKT4	++	++	++	++	++	++
B9.11	++	++	++	++	++	++

(a) Schematic representation of the inhibition of binding of 50 ng <sup>125</sup>I-labelled anti-CD4 mAb to 10<sup>6</sup> CEM cells by an excess ( $5 \times 10^3$  ng) of various anti-CD4 and negative control (B9-11) competitor mAb. Each score has been deduced from repeated titrations of a given inhibitor mAb in the range of 10<sup>-4</sup> ng to 10<sup>4</sup> ng. Controls have been determined as follows: the inhibition of binding obtained with 10<sup>4</sup> ng of the homologous cold competitor was considered 100 % inhibition, whereas the binding obtained using the same amount of an irrelevant mAb (B9-11) has been considered as 0 % inhibition.

++ refers to an absence of inhibition (binding of <sup>125</sup>I-mAb of more than 70 percent); + refers to partial inhibition, and — refers to complete inhibition (less than 30 % binding).

(b) CD4<sup>+</sup> cell lines (MT4 and CEM) were incubated with saturating amounts of competitor mAb as described in "Patients and Methods", exposed to HIV1-BRU and extensively washed. The effect of treatment was monitored by following RT activity (CEM) or cytopathic effect (MT4); — means absence of detectable infection on day 17 and ++ means positive RT and syncytia formation on day 7.

patient blood sample, the densities of these 5 CD4 epitopes per cell are statistically different from one another ( $p < 0.001$ , evaluated by a one-way Anova test), and a pairwise comparison permitted classification of these epitope densities on each cell as follows: OKT4A, 13B8-2, ST4#ST40, and BL4.

### DISCUSSION

The main purpose of this study was to quantify, by cytofluorometry, the surface density of five CD4 epitopes on lymphocytes from HIV-

infected patients at different stages of the disease.

Our data clearly demonstrate that the absolute number of CD4 molecules per cell in HIV-infected patient blood samples remains unchanged whatever the total number of CD4<sup>+</sup> cells per mm<sup>3</sup> and the severity of the disease. Moreover, each individual CD4 epitope density remains constant even when the p25 antigen is detectable in the samples analysed.

The steadiness in CD4 epitope density, which contrasts with the fall in CD4<sup>+</sup> cell count

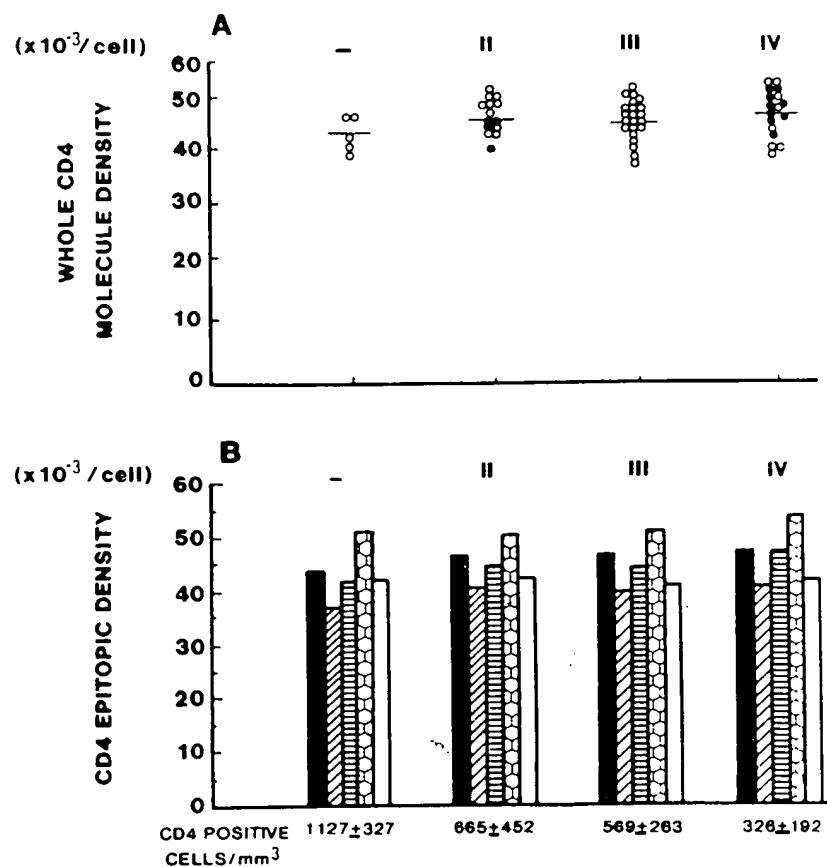


Fig. 2. Lymphocyte cell surface CD4 density in the course of AIDS.

A) CD4 density (mean number of CD4 molecules) per cell in HIV<sup>-</sup> individuals (—) and patients at various stages of AIDS (II to IV). The following symbols were used: ○ = patient with negative antigenaemia and ● = patient with positive antigenaemia.

B) Measurement of CD4 epitopic density per cell using 5 mAb: ■ = ST4; diagonal hatched column = OKT4A; horizontal hatched column = ST40; hexagonal hatched column = BL4, and □ = 13B8.2. The mean number of CD4<sup>+</sup> cells/mm<sup>3</sup>/patient is given.

throughout the progression of the disease, might be explained by the death of CD4<sup>+</sup> cells either due to HIV infection *per se*, syncytia formation, cytotoxic T cells, antibody-dependent cell cytotoxicity or other mechanisms suspected to cause a decrease in the number of circulating CD4<sup>+</sup> cells (reviewed in Fauci, 1988; Levy, 1989) without modifying the CD4 surface density of the surviving cells.

Second, the pattern of CD4 expression we observed here might have been due to a CD4 molecule downregulation from the membrane of infected cells. *In vitro* HIV infection is correlated with the disappearance of the CD4 receptor from the surface of the infected cells by a mechanism involving CD4-gp120 complex formation (Hoxie *et al.*, 1986) and/or regulation at the gene level (Salmon *et al.*, 1988). Yet the small percentage of HIV-infected cells evidenced *in vivo* (Psallidopoulos *et al.*, 1989) could not account for the drastic drop in the CD4<sup>+</sup> cell count observed in AIDS. Furthermore, HIV-mediated CD4 downregulation does not seem to be as systematic *in vivo* as it is *in vitro* (Schnittman *et al.*, 1989; Walker *et al.*, 1989).

The fact that we have not seen any variation in the density of either CD4 molecules or CD4 epitopes per cell among patient samples argues against any form of site-related hindrance or partial CD4 internalization, suggesting interference from circulating virus, soluble gp120 or self-reacting antibodies (Weinhold *et al.*, 1988; Kowalski *et al.*, 1989), considered as important physiopathological events (Weinhold *et al.*, 1988; Lanzavecchia *et al.*, 1988). This suggests that such phenomena are of too low a magnitude to be detected by the phenotyping approaches used in this study, if they do exist at all.

On the other hand, for each lymphocyte studied whatever its patient origin, slight but significant differences were constantly observed between the five individual epitopic densities measured. We observed the same variations in epitopic densities in tumour CEM cells (data not shown). Such differences have already been reported for some CD4 epitopes and are considered to be genetically determined (Parker *et al.*, 1988; Carrel *et al.*, 1988). Here, these variations did not seem to be genetically determined, since

each individual epitope density per cell remained constant throughout the different patient samples; nor were they related to affinity differences between the various anti-CD4 mAb, as revealed by Scatchard analysis (data not shown). Rather, they might result from steric hindrance of some epitopes on a low percentage of CD4 molecules involved in multimolecular cell surface complexes (Anderson *et al.*, 1988; Rivas *et al.*, 1988).

Finally, our observations suggest that any mAb reacting with either the V1 or V2 domain of CD4 may be used to follow the evolution of the CD4 cell count of HIV-infected patients by immunophenotyping. In addition, the count of CD4 molecules directly correlates with the count of CD4 lymphocytes in AIDS.

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#### La densité des molécules CD4 exprimées à la surface des lymphocytes de patients infectés par le VIH reste constante au cours de l'évolution de la maladie

Dans la mesure où de nombreux travaux suggèrent l'existence éventuelle de particules virales circulantes, de molécules gp120 solubles ou d'auto-anticorps anti-CD4 dans le sang périphérique des patients infectés par le VIH (virus de l'immuno-déficience humaine), nous avons cherché à estimer l'influence de ces paramètres sur le phénotypage lymphocytaire des patients séropositifs. Pour cela, cinq anticorps monoclonaux définissant différents épitopes portés par les domaines V1 et V2 de la molécule CD4 ont été utilisés pour l'analyse de la densité des molécules CD4 par lymphocyte chez des patients séropositifs classés dans les groupes II, III et IV, d'après les critères des « Centers for Disease Control » d'Atlanta (USA) pour chacun de ces anticorps.

Nous démontrons que la densité de surface de chaque épitope testé reste constante (avec une valeur moyenne de 46000 épitopes par lymphocyte) quelque soit le stade de la maladie et la concentration sérique d'antigène p25 chez les patients étudiés.

Ces résultats suggèrent que le pouvoir d'accès des anticorps à différents épitopes CD4 n'est en rien

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modifiée par des interactions entre ces molécules et la présence hypothétique de particules virales circulantes, de gp120 et/ou d'autoanticorps anti-CD4. Si de telles interactions existent *in vivo*, comme on pourrait le penser, leur importance est trop faible pour avoir des conséquences mesurables au niveau du phénotypage lymphocytaire.

Enfin, il est également montré que plusieurs anticorps monoclonaux dirigés contre les domaines V1 et V2 du CD4 peuvent être indifféremment utilisés pour le suivi biologique des individus infectés par le VIH.

**Mots-clés:** SIDA, VIH, CD4, Lymphocyte T; Phénotypage, Domaines V1-V2.

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## Human Immunodeficiency Virus-Infected Monocyte-Derived Macrophages Express Surface GP120 and Fuse with CD4 Lymphoid Cells *in Vitro*: A Possible Mechanism of T Lymphocyte Depletion *in Vivo*<sup>1</sup>

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Monocyte-derived macrophages (MDM) infected *in vitro* with a macrophage-tropic strain of human immunodeficiency virus (HIV) fused with uninfected, CD4-expressing T lymphoblastoid cells, but not with a subclone of these cells lacking surface CD4. Infected MDM also fused with uninfected autologous and heterologous MDM. Recombinant soluble CD4 protein (rsCD4) (10 µg/ml) and full-length recombinant glycosylated gp120 (20 µg/ml) each inhibited fusion by 94-99%; the inhibition was dose-dependent. The N-terminal portion of gp120 did not inhibit syncytium formation. Fusion was also inhibited by a monoclonal antibody to an epitope which binds gp120 (S3.5), but not by antibody to an epitope not involved in gp120 binding (OKT4). HIV-infected MDM specifically bound fluorescein-conjugated rsCD4, and virus could be visualized budding from the surface of these cells. HIV-infected MDM express viral gp120 on their surface and fuse with CD4-bearing cells in a fashion similar to lymphoid cells. Macrophages may contribute to CD4 lymphocyte depletion *in vivo* by this fusion mechanism. © 1992 Academic Press, Inc.

### INTRODUCTION

Gradual, progressive loss of CD4-bearing T lymphocytes is characteristic of human immunodeficiency virus (HIV) infection *in vivo* (1). Mechanisms proposed to account for this selective cellular depletion include

poorly defined cytopathic effects related to T cell activation (2), autoimmune phenomena (3), and HIV-induced cytopathology involving fusion between HIV-infected and uninfected CD4-bearing T lymphocytes (4). This fusion process is mediated by interactions between certain epitopes of the CD4 molecule on uninfected T lymphocytes (5) and specific domains within the HIV envelope glycoproteins (6, 7). Recently, adhesion molecules such as LFA-1 have been shown to play an adjunctive role in T cell fusion (8). Because only a minority of CD4-expressing T cells are found to be productively infected by *in situ* hybridization (9) or to contain HIV cDNA (10, 11), it is difficult to explain the marked depletion of this subset of lymphocytes solely on the basis of either HIV infection alone, or fusion between infected and uninfected CD4-expressing T cells.

HIV-infected monocytes and macrophages have been found in peripheral blood and tissues of HIV-infected individuals (12, 13-20). The proportion of peripheral blood monocytes and tissue macrophages infected *in vivo* remains uncertain, with up to 15% of tissue macrophages reported to be infected (12, 13-20). HIV-infected multinucleated giant cells have been demonstrated in brain tissue and spinal cords of HIV-infected individuals with subacute encephalopathy and myelopathy using immunohistochemical techniques and *in situ* hybridization (15, 20).

Studies by several groups have shown that up to 70% of MDM can be infected with HIV *in vitro* (17-19). To date there has been no information available concerning HIV envelope expression in infected MDM. One study reported that HIV budded almost exclusively into intracellular vacuoles within infected MDM (21). In this communication we provide several lines of ev-

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idence that HIV-infected MDM express functional HIV gp120 on their surface.

# METHODS

**Isolation of monocytes.** Monocytes were isolated from buffy coats of HIV seronegative donors (purchased from Stanford Blood Bank, Palo Alto, CA) by Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) density gradient centrifugation and glass adherence, as previously described (19). Briefly, the mononuclear cells were collected after density gradient centrifugation, washed, and resuspended in RPMI-1640 (GIBCO Laboratories, Grand Island, NY) supplemented with 20% heat-inactivated fetal calf serum (FCS, Hyclone Laboratories, Logan, UT). Approximately  $5 \times 10^8$  mononuclear cells in 20 ml media were placed in a sterile prewarmed 150-mm glass petri dish (Fisher Scientific, Pittsburgh, PA) and allowed to adhere for 2 hr at 37°C in a 5% humidified CO<sub>2</sub> incubator. The adherent monolayer was washed six times with warm phosphate-buffered saline (PBS) to remove non-adherent cells. The monolayer was then covered with calcium and magnesium free PBS (PBS-CMF, Cell Culture Facility, UCSF, CA), incubated on ice for 15 min, and then gently scraped from the dish. The cells were washed; resuspended in RPMI-1640 supplemented with 10% AB-positive human serum (HS, Stanford Blood Bank), 2 mM L-glutamine, and 50 µg gentamicin/ml; and placed in perfluoralkoxy jars (Saville, Minnetonka, MN) for maintenance in suspension culture, at a concentration of  $1 \times 10^6$ /ml. The culture medium was changed weekly.

The purity of monocyte preparations was assessed by nonspecific esterase staining ( $\alpha$ -naphthyl acetate esterase kit, Sigma Diagnostics, St. Louis, MO), LeuM3 staining (Becton-Dickinson, Mountain View, CA), and phagocytosis of yeast. Contaminating lymphocytes were removed by incubation of cells with OKT3 (Ortho Diagnostic Systems, Inc., Raritan, NJ) at a concentration of 2 µg/10<sup>6</sup> cells for 30 min at 4°C followed by incubation with a dilution 1:3 of stock complement solution (Cedarlane Laboratories, Hornby, Ontario) at 37°C for 45 min, as previously described (22). Viability was assessed by trypan blue exclusion.

The uninfected CD4-expressing (VB) and CD4 non-expressing (VB-CD4-negative) T lymphoblastoid cell lines have been described previously (4). The uninfected T lymphoblastoid H9 cell line and HIV-infected H9 cell line, HXB, were generously provided by Dr. R. Gallo (23). These cell lines were maintained in RPMI-1640 supplemented with 10% FCS.

**HIV infection of MDM.** HIV-1 DV strain, an isolate from the peripheral blood mononuclear cells of an HIV-infected male with Kaposi's sarcoma (provided by Dr. G. Reyes, GeneLabs, Redwood City, CA), was used to infect cultured MDM as previously described (19). The

stock viral suspension ( $10^{5.5}$  TCID<sub>50</sub>/ml, as assessed by VB cell cytopathology assay) was prepared in VB cells, stored in small volumes at -70°C, and thawed just prior to use. MDM were infected at a multiplicity of approximately one infectious particle per cell (calculated from the Poisson distribution, 1 TCID<sub>50</sub> = 0.6 infectious unit), for 24 hr at 37°C. After washing in PBS-CMF supplemented with 1% FCS, cells were resuspended in media and incubated in perfluoralkoxy jars.

**Assessment of HIV infection of monocyte-macrophages.** HIV p24 antigen expression within MDM was assessed by staining prefixed and permeabilized cells with murine monoclonal anti-p24 antibody, IgG<sub>1</sub>, (provided by Dr. J. Carlson, UC Davis, CA), or purified mouse myeloma protein as an irrelevant control antibody of same class and isotype, MOPC21 (Litton Biogenetics, Kensington, MD) as previously described (7). Quantitation of antigen expression was achieved by flow cytometry using an Ortho Cytofluorograf-2S (Ortho Diagnostics, Raritan, NJ) equipped with a biohazard containment flow cell. HIV-infected MDM cultures were assayed for HIV p24 antigen expression by immunocytofluorographic analysis 9-14 days after infection. Cultures in which greater than 25% of cells expressed p24 were used for fusion experiments.

**Fusion assays.** Stock solutions of 600 µg/ml fluorescein isothiocyanate (FITC, isomer I, Sigma Chemical Co., St. Louis, MO) and 30 µg/ml rhodamine isothiocyanate (RITC, isomer R, BBL) were prepared as previously described (4). Infected MDM and uninfected lymphoblastoid cell targets were labeled with FITC (120 µg/ml) or RITC (10 µg/ml) and washed as previously described (4). T lymphoblastoid cell and MDM cell pellets were resuspended in RPMI/10% HS at a concentration of  $10^{5.3}$  cells/ml, mixed at a 1:10 ratio (unless otherwise specified), and incubated at 37°C in 5% CO<sub>2</sub> in the dark for 48 hr in perfluoralkoxy containers. Following fixation with 1% formalin, cultures were examined by phase contrast epifluorescence microscopy and scored for numbers of bifluorescent multinucleated giant cells (greater than or equal to four nuclei contained within a common cell membrane). With each assay, fluorescein-labeled HXB cells were incubated with rhodamine-labeled uninfected lymphoblastoid cells as a positive control; uninfected H9 cells were similarly incubated with lymphoblastoid cells to provide a negative control. Experiments were also performed using fluorescein-labeled infected MDM and rhodamine-labeled uninfected autologous and heterologous MDM, as well as fluorescein-labeled HXB cells and rhodamine-labeled uninfected MDM in the ratios described above.

For transmission electron microscopy, representative cultures of HIV-infected MDM mixed with CD4-expressing lymphoblastoid cells were fixed with 3%

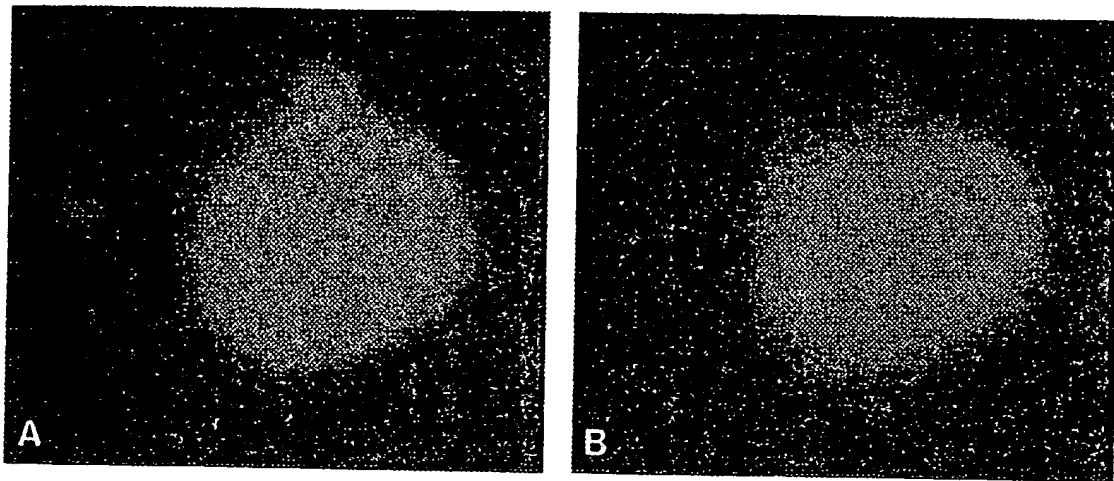


FIG. 1. HIV-infected MDM were treated with anti-OKT3 and complement to remove contaminating T lymphocytes, then labeled with fluorescein. Uninfected CD4-expressing T lymphoblastoid cells (VB cells) were labeled with rhodamine. Cells were incubated at a 1:1 ratio in the dark for 48 hr at 37°C, then syncytia were scored using a Zeiss Universal phase contrast fluorescence microscope. (A) (Filters set for rhodamine) A multinucleated giant cell containing VB lymphoblastoid cells. Note single lymphoblastoid cell not involved in fusion process which is therefore not observed in (B). (B) Same field but filter changed to observe green (fluorescein fluorescence). Multinucleated giant cell also contains HIV-infected MDM. (400×)

glutaraldehyde after 48 hr of cocultivation prior to embedding, sectioning, and staining. MDM and T cells were identified on the basis of their characteristic morphology; MDM have many cell processes, large cytoplasm-to-nucleus ratio, a well-developed Golgi apparatus, small amounts of glycogen and rough endoplasmic reticulum, and numerous lysosomes and enzyme-containing electron-dense granules; T cells have a large nucleus-to-cytoplasm ratio and contain relatively few intracytoplasmic structures.

**Fusion-inhibition assays.** Varying concentrations of murine monoclonal antibody S3.5, which recognizes an epitope of CD4 involved in binding gp120 (4), were incubated with  $10^5$  uninfected lymphoblastoid cells for 30 min in duplicate in LabTek slide chambers (Nunc, Inc., Naperville, IL). Equal numbers of HIV-infected MDM were then added to the lymphoblastoid cells and additional S3.5 was added to restore the initial concentration of antibody. Following incubation at 37°C for 48 hr, the numbers of syncytia in each of 20 low-power fields (10×) within each LabTek chamber were scored. Cell viability was assessed by trypan blue exclusion and was greater than 90% in all experiments. These figures were compared with results obtained in the control assay wells to which an irrelevant control antibody of the same class and isotype (MOPC21) had been added and with MDM incubated alone. Antibody to the adhesion molecule, LFA-1 (8), was similarly tested.

Recombinant soluble CD4 protein, representing the entire external domain of the molecule (generously provided by Dr. R. Sweet, SmithKline Beecham, King of Prussia, PA) (24), was incubated with infected MDM for 30 min prior to the addition of uninfected lympho-

blastoid cells. Additional CD4 protein was then added to maintain the desired final concentration and the cells were incubated in LabTek chambers for 48 hr. Controls to which no protein was added were included in each experiment.

Recombinant full-length glycosylated gp120 from HIV strain HTLV<sub>III</sub>B, prepared in *Drosophila* cells (25) (kindly provided by Dr. C. DeBouck, SmithKline Beecham), was incubated with uninfected lymphoblastoid cells for 30 min at 37°C, prior to the addition of infected MDM. Additional gp120 was added to maintain the desired concentration. A recombinant protein representing the N-terminal one third of HIV gp120 (also provided by Dr. C. DeBouck, SmithKline Beecham) was incubated with lymphoblastoid cells in separate wells as a control.

In every instance, each concentration of inhibitor

TABLE 1  
Kinetics of Fusion between VB Cells and HIV-Infected MDM

MDM:VB cell ratio	Number of multinucleated giant cells observed per replicate culture after indicated incubation times		
	24 hr	48 hr	72 hr
10:1	0, 0	3, 3	6, 8
1:1	4, 3	9, 7	97, 33
1:10	11, 10	100, 134	182, 146

Note. Fluorescein-labeled MDM ( $5 \times 10^4$ ) from two different donors were mixed with rhodamine-labeled VB lymphoblastoid cells ( $5 \times 10^3$ ,  $5 \times 10^4$  and  $5 \times 10^5$ ) and incubated in the dark in 5% CO<sub>2</sub>. After 24, 48, and 72 hr each culture was examined and scored for numbers of bifluorescent syncytia.

was assayed in duplicate, and each experiment was repeated on at least three occasions.

**Fluorescein conjugation of CD4.** Recombinant soluble CD4 was conjugated with FITC using a modification of previously published methods (26). CD4 was dialyzed overnight in cellulose dialysis tubing (Spectrapor 4, Applied Scientific, San Francisco, CA) at pH 9.5 in dialysis buffer. FITC in 1% dimethyl sulphoxide (Fisher Scientific, Pittsburgh, PA) was incubated with CD4 at a concentration of 100  $\mu$ g FITC/mg CD4 for 2 hr at 20°C in the dark. The conjugated CD4-FITC mixture was passed through a Sephadex G-25 column (Pharmacia LKB, Piscataway, NJ) containing 0.1% azide in PBS to remove unreacted or hydrolyzed FITC. The first colored fraction was collected.

**Demonstration of surface HIV gp120 on infected MDM.** About  $10^{5.5}$  HIV-infected MDM were washed in PBS-CMF/1% FCS/0.1% azide, and the cell pellet was incubated with fluorescein-conjugated CD4 (25  $\mu$ g/ml) for 30 min at 4°C. Following washing, cells were fixed in 0.5% formalin and analyzed by flow cytometry. Uninfected MDM and infected HXB and uninfected H9 cells were incubated with fluorescein-conjugated CD4 as controls.

## RESULTS

Purity of monocyte and MDM cultures as assessed by nonspecific esterase staining, phagocytosis of *Candida albicans*, and Leu-M3 staining was between 85 and 90%. Contaminating T lymphocytes, as assessed by immunocytofluorographic analysis using OKT3 monoclonal antibody, accounted for 3–5% of the population on initial isolation and less than 1% following OKT3/complement treatment, cultivation, and HIV infection.

**Fusion of HIV-infected MDM with uninfected lymphoblastoid cells.** When fluorescein-labeled, HIV-infected MDM were cultured with uninfected rhodamine-labeled CD4-bearing lymphoblastoid cells (VB cells), bifluorescent multinucleated giant cells were observed within 24 hr (Fig. 1). The numbers of giant cells observed in the control wells increased up to 10-fold between 24 and 48 hr of incubation, and about 2-fold between 48 and 72 hr (Table 1). At VB cell densities which were not limiting, over 100 syncytia were observed in each culture well containing  $5 \times 10^4$  MDM after 48 hr or more incubation. In parallel studies all multinucleated giant cells were positive for macrophage-associated nonspecific esterase (data not

shown). The bifluorescent syncytia were not due to phagocytosis of VB cells by MDM, since mixing fluorescein-labeled uninfected MDM and rhodamine-labeled CD4-bearing lymphoblastoid cells did not result in production of bifluorescent multinucleated giant cells. Infected MDM did not fuse with a subclone of the VB cells lacking surface CD4 (4) (data not shown), indicating that the CD4 molecule is necessary for the fusion process to occur. Fusion between infected MDM and lymphoblastoid cells was confirmed by direct visualization with transmission electron microscopy (Figs. 2a and 2b). Fusion between HIV-infected MDM and either autologous or heterologous uninfected MDM also occurred, although fewer syncytia were formed (average of 25 per  $10^5$  MDM) and the syncytia were smaller in size than those resulting from VB-MDM fusion.

**Inhibition of fusion by monoclonal antibodies and recombinant proteins.** Monoclonal antibody to CD4 (S3.5) inhibited fusion between HIV-infected MDM and VB cells in a concentration-dependent manner (Table 2). This antibody is directed against an epitope of CD4 that has previously been shown to be essential for fusion between HIV-infected lymphoid cells and uninfected CD4-bearing lymphocytes (4). OKT4 and an irrelevant control monoclonal antibody of same class and isotype (MOPC21) did not inhibit fusion. Monoclonal antibody LFA-1 also inhibited fusion between HIV-infected MDM and lymphoblastoid cells (Table 2). Fusion between HIV-infected MDM and lymphoblastoid cells was also blocked by recombinant soluble CD4 (Table 2). CD4-mediated inhibition of fusion was concentration-dependent with activity still detected at concentrations as low as 100 ng/ml (approximately  $2 \times 10^{-9}$  M). Fusion was inhibited by full-length recombinant HIV gp120, again in a dose dependent manner (Table 2) but not by the N-terminal third of the molecule tested at similar concentrations. Cell viability was unaffected by either rsCD4 or gp120.

**Demonstration of HIV gp120 on surface of infected MDM and HXB cells.** Cytofluorographic analysis showed that as compared with uninfected cells, both HIV-infected MDM and HXB cells bound fluorescein-labeled CD4 (Fig. 3). By electron microscopy, although virus was mainly visualized in intracellular vesicles within HIV-infected MDM, virus was also seen budding from the surface of the infected cells (Figs. 2c and 2d).

**FIG. 2.** Transmission electron micrographs of HIV-infected MDM fusing with CD4-expressing T lymphoblastoid cells (VB cells). A T lymphoblastoid cell (T) adherent to an HIV-infected MDM (M) is seen in (a). Examination at higher power demonstrates continuity between the plasma membranes of the two cells with an area of loss of individual cell membranes separating the cytoplasm of the two cells (b). Typical retroviral particles could be seen within intracytoplasmic vacuoles in the infected MDM (c). The virus is also visualized budding from the surface of the HIV-infected MDM (d).



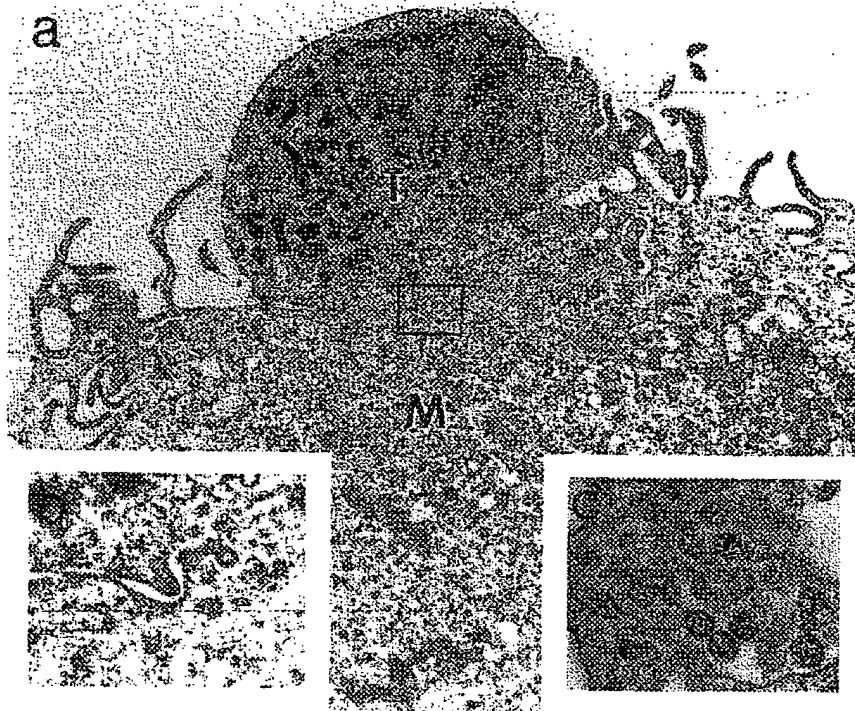


TABLE 2  
Inhibitors of Fusion between HIV-Infected MDM and  
CD4-Expressing Lymphoid Cells<sup>a</sup>

Test compound	Concentration (μg/ml)	Mean % inhibition of fusion (range)
Soluble CD4 <sup>b</sup>	50	>99 (98–100)
Soluble CD4	20	98 (97–100)
Soluble CD4	10	94 (90–95)
Soluble CD4	5	81 (79–84)
Soluble CD4	0.1	23 (10–32)
gp120 <sup>c</sup>	10	100 (100)
gp120	3	100 (100)
gp120	1	96 (93–99)
gp120	0.1	35 (33–41)
N gp120 <sup>d</sup>	15	6 (0–13)
S3.5 <sup>e</sup>	20	>99 (99–100)
S3.5	10	45 (40–59)
S3.5	0.1	0 (0–3)
anti-LFA-1	20	100 (100)
MOPC 21 <sup>f</sup>	20	0 (0)

<sup>a</sup> Chronically HIV-infected MDM were mixed with CD4-expressing (CD4<sup>+</sup>) VB lymphoblastoid cells in the fusion assay described (see Methods). In control wells to which no inhibitor was added the mean number of multinucleated cells was 110 (range 69 to 172) in 14 separate experiments.

<sup>b</sup> Recombinant full-length soluble CD4 (10).

<sup>c</sup> Full-length, recombinant glycosylated HIV gp 120 (26).

<sup>d</sup> Recombinant non-glycosylated N-terminal one third of HIV gp120.

<sup>e</sup> Anti-CD4 monoclonal antibody.

<sup>f</sup> Irrelevant monoclonal antibody control of same class and isotype as S3.5.

## DISCUSSION

HIV infection of MDM has been clearly documented both *in vitro* and *in vivo* (17–20, 23, 27). In this communication we show that HIV envelope glycoprotein is present on the surface of HIV-infected peripheral blood MDM, as it is on T cell lines (4, 28, 29). MDM surface gp120 bound CD4 on uninfected cells and thereby initiated fusion with these cells (30). The fusion process was inhibited by soluble recombinant CD4 and full-length recombinant gp120, as well as by antibodies directed against the epitope of CD4 that binds HIV gp120 (5, 30). The recombinant N-terminal portion of gp120, a part of the protein not primarily involved in binding with CD4 (6, 28), did not inhibit fusion, nor did monoclonal antibody OKT4, which is directed against a region of CD4 distant from the binding site (5, 30).

The ability of HIV-infected MDM to fuse *in vitro* with uninfected CD4-bearing T lymphoid cells provides a potential mechanism for the gradual but progressive depletion of the CD4 subset of T lymphocytes *in vivo* characteristic of patients with HIV infection (1). Although multinucleated giant cells are not frequently encountered in tissues from infected patients, there are specific areas (for example, the brain and spinal cord of patients with HIV-related encephalopathy and myelopathy) where syncytia are found which have the his-

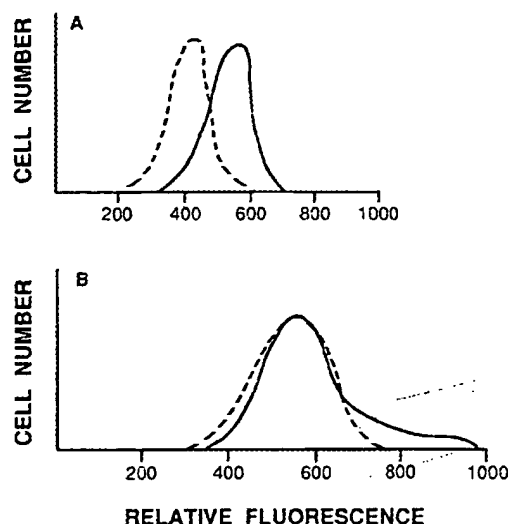


FIG. 3. Tracing of cytofluorographic analysis of H9 lymphoblastoid cells (A) and MDM (B) stained with fluorescein-conjugated recombinant soluble CD4. Uninfected (---) and HIV infected (—) tracings are shown for each cell type.

tologic and cytochemical markers of macrophages (15, 16). Macrophage-containing multinucleated giant cells are present in the lungs of cats infected with a related virus, feline immunodeficiency virus (P. Luciw, personal communication).

Our data show that HIV-infected MDM express cell surface HIV envelope determinants. While HIV has been shown to bud in abundance from the surface of infected T cells, Gendelman *et al.* reported that the virus was localized predominantly within intracytoplasmic vesicles of MDM (21), and budding from the surface of the cell has been questioned. The presence of HIV gp120 on the surface of HIV-infected MDM and T cells has been difficult to demonstrate directly. Surface staining of HIV-infected MDM using a variety of anti-gp120 monoclonal antibodies has often been unsatisfactory, although these same cells will bind monoclonal antibodies to HIV gp120 following fixation and permeabilization (31; Crowe *et al.*, unpublished data). However, using fluorescein-conjugated CD4 we could detect the presence of surface gp120 on both MDM and T cell lines. This indirect evidence for surface gp120 is supported by electron micrographs showing virus budding from the surface of the infected macrophage, as well as within intracytoplasmic vesicles. It would appear that gp120 is not detectable on the surface of all infected cells, as the proportion of cells that stain with anti-HIV p24 monoclonal antibody is higher than the proportion staining with labeled-CD4 (31; Crowe *et al.*, unpublished data). This may be because the prefixation and permeabilization steps used to detect intracytoplasmic p24 expose more antigen than surface staining for gp120 (31). Alternatively, the surface ex-



pression of gp120 may be too low to demonstrate by immunocytofluorographic analysis in some cells, perhaps because of formation of gp120-CD4 aggregates retained intracellularly (32-34).

Our data showing surface expression of gp120 on MDM are not in accord with published studies (31). Using a MAb that reacts with both gp160 and gp120, Potts and colleagues did not identify envelope glycoproteins on the surface of infected MDM (31). One possible reason for the discrepant data between this group and ours may be the differing culture systems employed. We add no cytokines or growth factors apart from those present in heat-inactivated 10% human serum. Potts and colleagues use M-CSF (1000 U/ml) in addition to 10% fresh human serum to supplement their growth media. Using this system they also find that most monocytes cultured in suspension for 15 days do not express surface CD4, an observation which again differs from ours (19).

As only rare T lymphocytes are productively infected with HIV *in vivo* (10, 11), and this infection is usually cytolytic *in vitro*, fusion between infected and uninfected T lymphocytes is unlikely to contribute substantially to CD4 lymphocyte depletion *in vivo*. On the other hand, MDM can be chronically infected with HIV, and the infected cells remain viable in culture for several months (19, 21). Through a cell fusion process such persistently infected monocytes and MDM may play a role in the inexorable removal of CD4 lymphocytes from the *in vivo* pool which is characteristic of HIV infection (1). The fact that infected MDM will also fuse with other CD4-expressing uninfected macrophages and remain viable for up to 2 weeks *in vitro* MDM would potentially allow continuing maintenance or even expansion of the *in vivo* reservoir of infected cells.

Fusion between infected MDM and the VB lymphoblastoid cells occurred more rapidly (within 24 hr) than fusion between infected and uninfected MDM (within 48 to 72 hr). This is likely to be related to the higher density of cell surface CD4 expressed by the uninfected T lymphoblastoid cell line compared to MDM (S. Crowe, unpublished data). Although 25 to 80% of uninfected MDM express surface CD4 (19, 27), the amount of CD4 present on monocytes (as assessed by quantitative immunocytofluorographic analysis) is less than one tenth of the amount of CD4 present on helper-inducer lymphocytes (27; S. Crowe, unpublished observations). Both the ability to induce cytopathology and susceptibility to infection have been found to correlate with density of surface CD4 expression (11, 35).

Although up to 80% of cultured MDM can be infected with HIV [as detected by anti-HIV p24 monoclonal antibody staining, fluorescein-conjugated CD4 staining, and flow cytometric analysis (19)], only about 0.5% of MDM fused with uninfected T lymphoblastoid cells in

our system. There are numerous possible reasons for this discrepancy. The short incubation times used for these assays, needed to avoid secondary fusion between T cells, may not permit maximal MDM-T cell fusion. Furthermore, an infected MDM probably has to fuse with multiple VB cells before it can be recognized as a giant cell. Estimation of the proportion of infected MDM is therefore limited by the maximum VB cell:MDM ratio which is feasible, about 10:1. Fusion may not occur with MDM expressing low concentrations of surface gp120 (7).

Fusion between infected and uninfected lymphocytes has been shown to be blocked by murine monoclonal antibodies directed against CD4 (4). Although these monoclonal antibodies inhibited fusion of infected MDM with lymphocytes equally well, the therapeutic potential of such antibodies is low, as a blockade of CD4 receptors throughout the host in itself has produced severe immunodeficiency (36). Administration of anti-CD4 antibody to patients with rheumatoid arthritis resulted in a decline in CD4<sup>+</sup> lymphocyte counts, decreased numbers of circulating monocytes, and suppression of delayed-type hypersensitivity reactions (37, 38). Although efficacious in inhibiting cellular fusion, recombinant HIV gp120 would likely cause the same adverse effects as anti-CD4 (3, 39). Inhibition of fusion between HIV-infected T cells by monoclonal antibodies directed against the adhesion molecule LFA-1 has been recently reported (8), and similar concentrations of this antibody inhibited MDM-T cell fusion in our experiments. LFA-1 may act as a secondary receptor involved in the fusion process (8). Soluble, recombinant full-length CD4 protein blocks binding of cell-free HIV to the cellular receptor (24). Our studies have shown that this protein can also prevent MDM-related fusion and transmission of HIV mediated by this mechanism. As spread of HIV infection *in vivo* involves both cell-associated virus and free virus, the ability of soluble CD4 to prevent cell fusion may be of therapeutic relevance.

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## Construction and Enhanced Cytotoxicity of a [Cyanovirin-N]-[*Pseudomonas* Exotoxin] Conjugate against Human Immunodeficiency Virus-Infected Cells<sup>1</sup>

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Cyanovirin-N (CV-N) is a novel 11-kDa anti-HIV (human immunodeficiency virus) protein that binds with high affinity to the viral envelope glycoprotein gp120. In contrast to soluble CD4 and most known neutralizing antibodies that bind gp120, CV-N exerts potent anti-viral activity against primary clinical HIV isolates as well as laboratory-adapted strains of HIV. Here we describe the recombinant production, purification, and characterization of a chimeric toxin molecule, FLAG-CV-N-PE38, that contains CV-N as a gp120-targeting moiety linked to the translocation and cytotoxic domains of *Pseudomonas* exotoxin A. FLAG-CV-N-PE38 showed enhanced cytotoxicity to HIV-infected, gp120-expressing H9 cells compared to uninfected H9 cells. Competition experiments with free CV-N provided further support that the enhanced FLAG-CV-N-PE38-induced cytotoxicity was due to interactions of the CV-N moiety with cell surface gp120. This study establishes the feasibility of use of CV-N as a gp120-targeting sequence for construction and experimental therapeutic investigations of unique new chimeric toxins designed to selectively destroy HIV-infected host cells. © 1997 Academic Press

Infection of most susceptible host cells by human immunodeficiency virus type 1 (HIV-1), or the related primate immunodeficiency viruses HIV type 2 (HIV-2) and simian immunodeficiency virus (SIV), begins with an initially reversible interaction of the viral envelope surface glycoprotein gp120 (gp120) with the cell surface receptor CD4 (CD4) (1). This is followed by virus-cell fusion and viral entry into the cell using a co-receptor belonging to the seven-transmembrane-spanning, G-protein-coupled receptor family (2, 3). Productively infected, virus-producing cells express gp120 at the cell surface. Interaction of gp120 on infected cells with CD4 on other infected or uninfected cells results in cellular fusion, formation of dysfunctional multinuclear cell masses (syncytia), and further spread of viral infection (4, 5).

Considerable attention has been devoted to recombinant CD4 proteins (soluble CD4 or sCD4) and derivatives thereof for potential applications in specific, gp120-targeted therapeutics. Interest in sCD4 was stimulated initially by observations of its binding to gp120 and inhibition of infectivity of laboratory-adapted strains of HIV *in vitro* (6-14). However, fresh clinical isolates of HIV, and likewise HIV in patients, have proven highly resistant to gp120-targeted inhibition by sCD4 and other sCD4-based therapeutics, including sCD4-coupled toxins designed to selectively bind and destroy HIV-infected, gp120-expressing host cells (15-22).

The discovery and initial characterization of cyanovirin-N (CV-N), a novel 11-kDa anti-HIV protein from a cultured cyanobacterium (*Nostoc ellipsosporum*), was recently reported (23, 24). Low nanomolar concentrations of CV-N irreversibly inactivate T-lymphocyte-tropic, macrophage-tropic and dual-tropic primary clinical isolates of HIV-1 as well as laboratory-adapted strains of HIV-1, HIV-2, and SIV *in vitro*. Furthermore,

<sup>1</sup> This is part 40 in the series HIV-Inhibitory Natural Products; for part 39, see reference 29.

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Abbreviations: HIV, human immunodeficiency virus; HIV-1, HIV type 1; HIV-2, HIV type 2; SIV, simian immunodeficiency virus; gp120, HIV envelope surface glycoprotein gp120; CD4, cell surface CD4 receptor; sCD4, soluble CD4; CV-N, cyanovirin-N; PE, *Pseudomonas* exotoxin; SDS-PAGE, sodium dodecyl sulfate/polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; F-CV-N, FLAG-CV-N; F-CV-N-PE38, FLAG-CV-N-PE38; *E. coli*, *Escherichia coli*; IPTG, isopropyl-1-thio- $\beta$ -D-glactopyranoside; HPLC, high-performance liquid chromatography; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin.

CV-N prevents *in vitro* fusion and transmission of HIV-1 between infected and uninfected cells. The anti-HIV activity of CV-N is mediated, at least in part, through high-affinity interactions with the viral envelope glycoprotein gp120 (23, 25). There are significant differences between CV-N, sCD4, and anti-gp120 monoclonal antibodies with respect both to the specificity of gp120 molecular interactions and their antiviral activities. In particular, CV-N is fully active against clinical isolates of HIV (23), and therefore may lack the "Achilles heel" which has frustrated attempts to clinically exploit sCD4 and derivatives thereof, including cytotoxic conjugates such as *Pseudomonas* exotoxin (PE) molecules linked to sCD4 (15-22).

To evaluate the potential utility of CV-N in targeting cytotoxic molecules to HIV-infected, gp120 expressing cells, we have created and characterized an unique new chimeric toxin molecule as described below.

## MATERIALS AND METHODS

**Materials.** Restriction endonucleases and T4 DNA ligase were obtained from Pharmacia Biotech (Uppsala, Sweden). *Pfu* DNA polymerase was obtained from Stratagene (La Jolla, CA). Reagents for sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) were obtained from Bio-Rad (Hercules, CA). All other chemicals were analytical reagent grade. The plasmid vector pRK79 (26) and anti-PE polyclonal antibodies (27) were as described.

**Oligonucleotides and amplification.** Oligonucleotides L1 (5'-CGA CGG TAC CCT GAA ACA ATT GTA CGA ACT CGA GTA ACT C-3'), L2 (5'-GAG TTA CTC GAG TTC GTA CAA TTG TTT CAG GGT ACC GTC G-3'), L3 (5'-ATC GAC AAG CTT GGT ACC CTG AAA TAC GAA GGC GGC AGC CTG GCC GCG CTG ACC GCG CAC CAG GCT TGC CAC CTG CCG CTG GAG ACT TTC ACC CGT CAT CGC GAG-3'), and L4 (5'-CTG CGG ATG ACG GGT GAA AGT CTC CAG CGG CAG GTG GCA AGC CTG GTG CGC GGT CAG CGC GGC CAG GCT GCC GCC TTC GTA TTT CAG GGT ACC AAG CTT GTC GAT-3') were prepared, as described previously (25). Polymerase chain reaction (PCR) and purification of the amplified fragments were carried out as described previously (25).

**Plasmids, bacterial strains, and cell lines.** Construction of the plasmid pPBS7 which encodes FLAG-CV-N (F-CV-N), a fusion protein with the eight amino acid FLAG leader sequence (AspTyrLys-AspAspAspAspLys) (28) joined to the NH<sub>2</sub>-terminus of CV-N, and plasmid pPBS7(-) which encodes for native CV-N, was described previously (23, 29). The plasmid pPBS7-L was constructed by insertion of a *Kpn*I-*Xho*I digested L1/L2 hybridized double-strand fragment into a *Kpn*I-*Xho*I fragment of plasmid pPBS7. Plasmid pUC19-L-PE38 was constructed by insertion of a *Hind*III-*Bsp*MI digested L3/L4 hybridized double-strand fragment and a *Bsp*MI-*Eco*RI fragment of plasmid pRK79 into a *Hind*III-*Eco*RI fragment of plasmid pUC19. The plasmid pPBS7-PE38 encoding FLAG-CV-N-PE38 (F-CV-N-PE38) was constructed by insertion of a *Kpn*I-*Eco*RI fragment of plasmid pUC19-L-PE38 into a *Kpn*I-*Mfe*I fragment of plasmid pPBS7-L. All of the plasmids were propagated in *Escherichia coli* (*E. coli*) DH5 $\alpha$  F'IQ (Gibco BRL, Gaithersburg, MD). The contents of each plasmid construct were confirmed by DNA sequencing.

**Expression of F-CV-N-PE38 in *E. coli*.** The plasmid pPBS7-PE38 was transfected into *E. coli* and the F-CV-N-PE38 conjugate protein was induced with isopropyl-1-thio- $\beta$ -D-galactopyranoside (IPTG) and expressed, as described previously (25). The recombinant protein was secreted into the periplasmic space of *E. coli* due to the presence of

the *ompA* signal peptide sequence (30). The structure of F-CV-N-PE38 is shown schematically in Fig. 1.

**Protein purification.** The expressed proteins were initially purified by affinity chromatography on agarose linked with an anti-FLAG monoclonal antibody, as described previously (23). The concentrated samples were further purified by high-performance liquid chromatography (HPLC) on a 2.15  $\times$  60 cm G3000PW gel permeation column (TOSOHAAS, Montgomeryville, PA), using a BioCAD SPRINT perfusion Chromatography System (PerSeptive Biosystems, Cambridge, MA) eluted with the equilibration buffer (400 mM NaCl in 50 mM sodium phosphate buffer, pH7.5) at a flow rate of 5 ml/min. Column operation was monitored by absorbance at 210 nm. The peak corresponding to the target protein was collected, filtered through a 0.45  $\mu$ m filter, and stored at -70°C. The proteins corresponding to CV-N and F-CV-N were purified as described previously (23, 29). All isolated proteins were homogeneous as judged by SDS-PAGE.

**SDS-PAGE analysis and Immunoblotting.** The purified samples were analyzed on 16.5% Tris-Tricine gels (Bio-Rad, Hercules, CA) by SDS-PAGE under reducing conditions. For immunoblotting, the purified samples were transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH) following SDS-PAGE, according to standard procedures. Blotting was performed with a mouse monoclonal antibody to the FLAG peptide (International Biotechnologies, Inc., New Haven, CT), rabbit polyclonal antibodies to CV-N (23), and rabbit polyclonal antibodies to PE (27). The respective detection antibodies were conjugated with horseradish peroxidase (Sigma, St. Louis, MO).

**Anti-HIV assays.** H9 cells and H9/HIV-1<sub>IIIB</sub> cells, chronically infected with HIV-1 (31) were used. Cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum. H9 and H9/HIV-1<sub>IIIB</sub> cells (5 $\times$ 10<sup>4</sup>/well) were cultured in 96-well microtiter plates in the absence or presence of various concentrations of F-CV-N-PE38 chimeric protein. After three days in culture, cellular viability was assessed using an XTT-tetrazolium assay as described by Gulakowski, et al (32).

**Studies of interactions of F-CV-N-PE38 with gp120.** An enzyme-linked immunosorbent assay (ELISA) was used to determine the relative binding of F-CV-N-PE38 and CV-N to gp120, as described previously for CV-N (23). Log<sub>10</sub> serial dilutions of each protein were added either to gp120 (100 ng/well) or to 1% bovine serum albumin (BSA)-coated control wells (one pmole of CV-N and F-CV-N-PE38 corresponds to 11 ng and 49.3 ng, respectively). The binding affinity of anti-CV-N antibodies to CV-N and to F-CV-N-PE38 was confirmed to be nearly identical.

## RESULTS AND DISCUSSION

We constructed an *E. coli* expression plasmid (pPBS7-PE38) containing a chimeric gene encoding FLAG-CV-N, plus amino acids 253-364 and 381-613 of PE (Fig. 1). This segment of PE (designated PE38, ref. 27 and 33) lacks domain Ia, the cell recognition domain, and part of domain Ib. It includes domains II and III, which are responsible for translocation and ADP-ribosylation, respectively. Induction of gene expression with IPTG resulted in secretion of the hybrid protein, designated F-CV-N-PE38, into the *E. coli* periplasm. The protein was isolated from periplasmic extracts by affinity chromatography on agarose linked with an anti-FLAG monoclonal antibody and then purified by gel filtration chromatography. SDS-PAGE and immunoblot analysis with anti-FLAG, anti-CV-N, and anti-

## Protein name

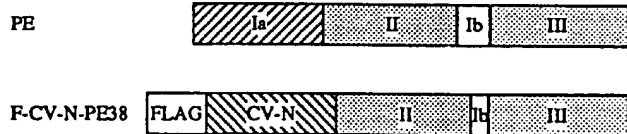


FIG. 1. Schematic representation of F-CV-N-PE38 constructed in this study. Full-length *Pseudomonas* exotoxin (PE) consists of domain Ia (amino acids 1-252), domain II (amino acids 253-364), domain Ib (amino acids 365-399), and domain III (amino acids 400-613). In the F-CV-N-PE38 conjugate, domain Ia was removed from the N-terminus and replaced with FLAG-CV-N and amino acids 365-380 were deleted from domain Ib.

PE antibodies of the purified material revealed a single band with the expected molecular mass of approximately 49.3 kDa (Fig. 2).

The human T-cell line H9 chronically infected with HIV-1<sub>IIIB</sub> (H9/HIV-1<sub>IIIB</sub>) is known to express viral envelope glycoprotein gp120 on the cell surface (31, 34). Therefore, the cytotoxicity of F-CV-N-PE38 on uninfected and infected H9 cells was investigated using an XTT-tetrazolium cell viability assay. As illustrated in Fig. 3A, H9/HIV-1<sub>IIIB</sub> cells were killed by F-CV-N-PE38 at a significantly lower concentration ( $IC_{50}=0.004$  nM) than uninfected H9 cells ( $IC_{50}=0.3$  nM), consistent with the view that cell binding and toxicity of the toxin was enhanced by the presence of cell-surface expressed gp120.

In a subsequent experiment, H9/HIV-1<sub>IIIB</sub> cells were treated with F-CV-N-PE38 in the presence or absence of free CV-N (91 nM). As shown in Fig. 3B, CV-N competed with F-CV-N-PE38 and reduced its level of toxicity to that of uninfected H9 cells. Addition of exogenous

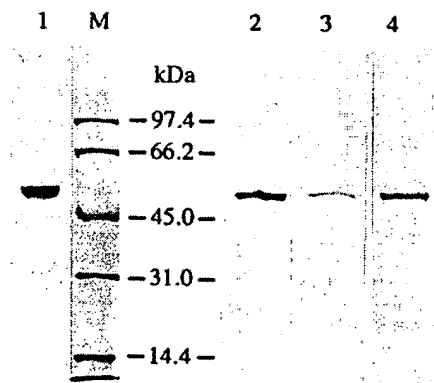


FIG. 2. SDS-PAGE and immunoblot analysis of purified F-CV-N-PE38. Gels (16.5% Tris-Tricine) were stained with Coomassie blue (lane 1, 1.0  $\mu$ g of protein) or immunoblotted with monoclonal antibody to FLAG peptide (lanes 2, 10 ng) or polyclonal antibodies to CV-N (lane 3, 50 ng) or PE (lane 4, 50 ng). Lane M, molecular weight standards.

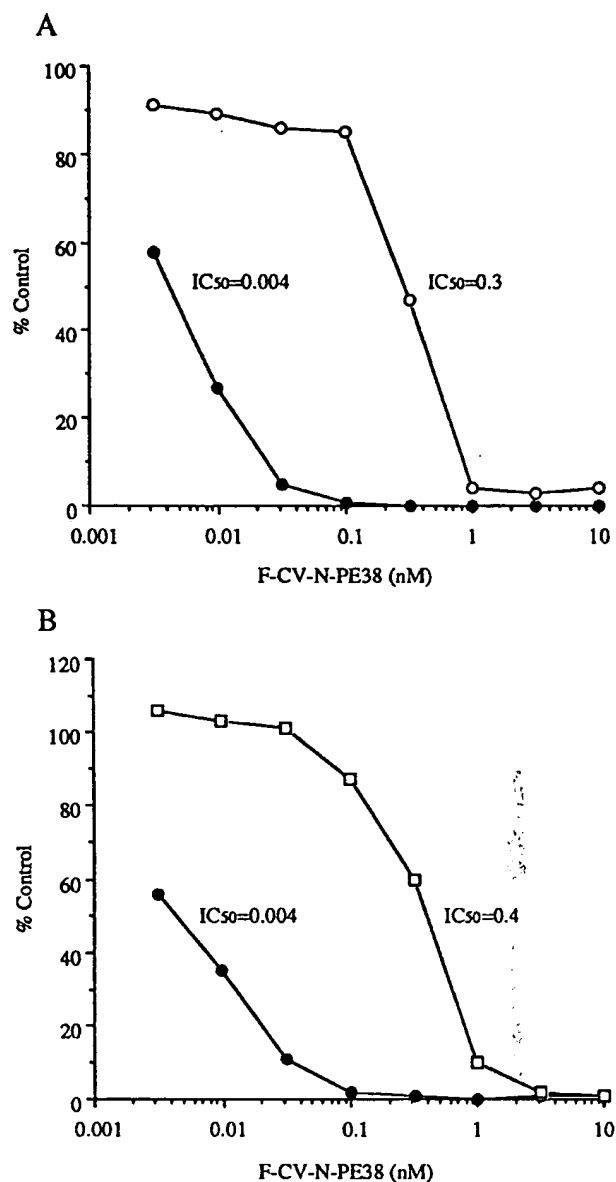
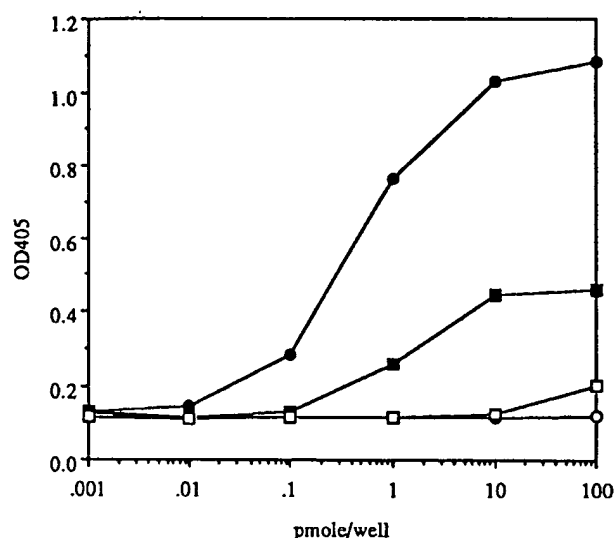


FIG. 3. Selective cytotoxicity of F-CV-N-PE38 for cells expressing the HIV envelope glycoprotein gp120. (A) XTT assay for detection of F-CV-N-PE38-mediated cell killing of uninfected H9 cells (○) and infected H9/HIV-1<sub>IIIB</sub> cells (●). (B) XTT assay for detection of F-CV-N-PE38-mediated cell killing of H9/HIV-1<sub>IIIB</sub> cells in the presence (□) or absence (●) of 91 nM CV-N. Points are averages of duplicate determinations and are expressed as percent control of untreated cultures.

FLAG peptide itself had no effect on the cytotoxicity of F-CV-N-PE38 (data not shown). This competition experiment provided additional evidence that a specific interaction of gp120 and the CV-N portion of the hybrid toxin is responsible for enhanced cell binding and subsequent cell killing by the conjugate.

The ability of F-CV-N-PE38 to bind gp120 was further assessed in an ELISA assay (Fig. 4). Both CV-N



**FIG. 4.** ELISA study of concentration-dependent binding of CV-N and F-CV-N-PE38 to gp120. CV-N (circles) or F-CV-N-PE38 (squares) at log<sub>10</sub> serial dilutions was added either to 100 ng/well gp120 (●, ■) or to BSA-coated control wells (○, □). Anti-CV-N polyclonal antibodies were used to detect the bound CV-N as indicated by absorbance at 405 nm. Points are averages of duplicate determinations.

and F-CV-N-PE38 proteins specifically bound to gp120-treated microtiter plate wells in a concentration-dependent manner, although F-CV-N-PE38 had somewhat less binding activity than CV-N. Neither the FLAG peptide alone nor a FLAG-PE38 fusion protein, which did not possess CV-N, showed detectable binding to gp120 (data not shown).

The lower gp120-binding affinity of F-CV-N-PE38 relative to CV-N may be due to steric hindrance of the CV-N-gp120 interactions and/or to changes in the tertiary structure of the CV-N moiety. Therefore, a variety of approaches for improving the gp120-targeting and selective cytotoxic effects of a CV-N conjugate may be possible. For example, insertion of appropriately modified "linker" peptide sequence(s) between CV-N and the toxin might provide a chimeric protein(s) with gp120 binding properties at least equivalent to those of unconjugated CV-N. Alternatively, it may be possible to covalently link low molecular weight, non-proteinaceous cytotoxic agents directly to CV-N that would not diminish CV-N's gp120-targeting attributes and thereby optimize selectivity of killing of HIV-infected versus noninfected cells by such a conjugate.

In conclusion, here we have shown that the attachment of CV-N to a PE38 exotoxin can confer gp120 binding activity and selectivity for killing of HIV-infected cells. Perhaps not surprisingly, conjugation of the relatively small CV-N protein to the much larger PE38 protein resulted in diminution of the overall gp120 binding activity. However, this study nonethe-

less demonstrates feasibility for further pursuit of the concept of CV-N-based, gp120-targeted killing of HIV-infected cells. CV-N inactivates diverse clinical isolates of HIV-1, regardless of their cellular tropism, as well as laboratory-adapted strains of HIV-1 and HIV-2 (23). It is apparent that CV-N binds to diverse gp120 molecules, despite extensive sequence variations in this viral envelope glycoprotein. Therefore, CV-N-based toxins may have distinct advantages over sCD4-based or monoclonal antibody-based toxin conjugates which selectively kill cells infected with laboratory-adapted strains but not primary isolates of HIV. The potential therapeutic value of this unique new class of cytotoxic reagent in the treatment of AIDS warrants further investigation.

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## CORRESPONDENCE

# CD4<sup>+</sup> T Cell Surface CCR5 Density and Virus Load in Persons Infected with Human Immunodeficiency Virus Type 1

To the Editor—Although CCR5 density may play a role in human immunodeficiency virus (HIV) disease progression, we suggest that the relationship between CCR5 density and virus load is more complex than was proposed recently by Reynes et al. [1]. The authors' conclusion that this relationship is independent of cellular activation was based on measurements of a single activation marker, HLA-DR, which has not been strongly associated with virus load or disease progression. Other factors of importance to HIV pathogenesis (e.g., cellular immune responses to HIV, HLA types, CCR2b, and viral characteristics) might have influenced the study's findings. Furthermore, the conclusion of Reynes et al.—that CCR5 density is independent of cellular activation—is in conflict with that of Husman et al. [2], who argued that CCR5 expression increased with HIV disease progression as a result of immune activation. The third figure and the text on page 929 in the article by Reynes et al. [1] support Husman et al.'s view, in that they report a significantly higher density of CCR5 on DR<sup>+</sup> than on DR<sup>-</sup> CD4<sup>+</sup> cells.

The small number of HIV-infected patients analyzed by Reynes et al. [1] (14 not undergoing highly active antiretroviral therapy [HAART] and 6 undergoing HAART) prompts caution in the extrapolation of their conclusions to the general population. Also, analysis of figure 1A of this article suggests that an "outlier," the right-most data point in the plot, may have skewed the statistical analysis. Without this observation, the Spearman rank correlation coefficient between plasma virus load and CCR5 density on all CD4<sup>+</sup> T cells increases from  $r = .666$  (see page 929) to  $r = .769$  ( $P = .004$ ), a value similar to the correlation coefficient,  $r = .749$  ( $P = .002$ ), between virus load and CCR5 density on DR<sup>-</sup>CD4<sup>+</sup> T cells. (The latter correlation coefficient also increases, albeit slightly, to  $r = .77$  with deletion of the same data point.) Thus, if this 1 data point is a true outlier, is in error, or represents a patient who differed from others in some important, unanalyzed way, then the correlation from the remaining data suggests that CCR5 expression is influenced by immune activation, and it would contradict the authors' assertion (page 930), "We found a correlation between virus load and CCR5 density on DR<sup>-</sup>CD4<sup>+</sup> T cells that was higher ( $r = .749$ ,  $P = .002$ ) than the correlation between virus load and CCR5 density for the whole CD4<sup>+</sup> T cell population (DR<sup>+</sup> and DR<sup>-</sup>). . . ." Without the outlying observation (see their second figure, panel B), the correlation coefficient between virus load and CCR5 density on DR<sup>+</sup> cells ( $r = .75$ ) is similar to that between virus load and CCR5 density on DR<sup>-</sup> cells ( $r = .77$ ).

The elimination of the outlier observation from figure 1A also is appealing, because it results in an improved log-linear

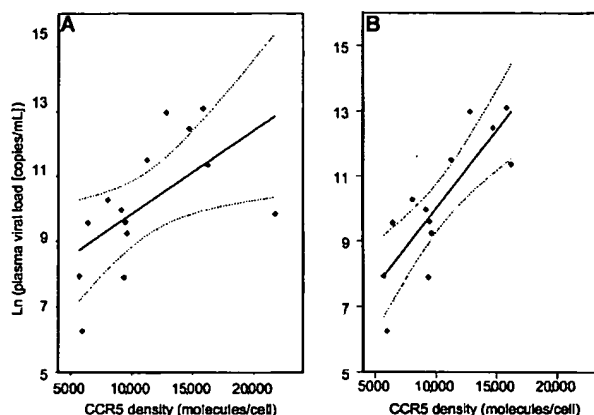


Figure 1. A, Complete data from figure 1A of Reynes et al. [1], with fitted regression line and 95% confidence interval; B, the same data, fit, and 95% confidence interval, less 1 outlying observation.

regression fit to the data with a lesser y-intercept: virus load =  $\exp[5.2 + 4.80 \times 10^{-4} \times (\text{CCR5 density})]$  (compare figures 1A and 1B, with and without the outlier observation, respectively). This equation explains 69% of the variance in virus load, in contrast to 35% of the variance when all 14 observations are included, and predicts a virus load in plasma of 182 copies/mL (95% confidence interval [CI], 23–1448 copies/mL) among persons with zero CCR5 density, that is, those who are homozygous for the 32 bp deletion, in contrast to 1408 copies/mL (95% CI, 131–15,087 copies/mL). The log-linear modeling prevents a prediction of zero virus load at zero CCR5 density. The regression model without the outlier observation, thus, is more in keeping with the unobservable virus load, and presumably unsustainable HIV-1 infection, among persons homozygous for the 32 bp deletion.

Finally, we question the conclusion of Reynes et al., from their fifth figure, that CCR5 density decreases transiently with immune activation. First, this experiment appears to be based on only 1 sample of peripheral blood lymphocytes, infected with 1 R5-tropic HIV isolate. Second, their use of relative densities (i.e., "percentages of densities expressed on control uninfected cells cultured concurrently"), rather than an absolute scale, on the left y-axis in this figure is troublesome. Last, their results in this experiment may be due to their choice of phytohemagglutinin (PHA) to stimulate cells. Bleul et al. [3] have shown that interleukin-2 is a much more efficient and perhaps a more physiological stimulator of CCR5 density than PHA.

In sum, we applaud Reynes et al.'s efforts and complete reporting of data, which allowed us to further analyze their results. We question, however, their analyses and interpretations, and we urge further research on the relationships among CCR5 density, immune activation, and HIV-1 virus load.

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#### Reply

**To the Editor**—We are grateful to Marmor et al. [1] for their comments on our article that was recently published in the *Journal of Infectious Diseases* [2]. They are right in their remark that cell activation, CCR5 expression, and human immunodeficiency virus type 1 (HIV-1) production are intricately related. This was the reason for our effort to establish that CCR5 density is linked to virus load independently of the level of cell activation. The result of this effort was the observation that CCR5 density at the surface of nonactivated HLA-DR<sup>-</sup>CD4<sup>+</sup> T cells is strongly correlated with HIV-1 viremia. It seems, however, that our argument has been misunderstood. Our conclusion was not that CCR5 density is independent of cellular activation. We even stated on page 929, lines 15–16, and showed in figure 3 that cellular activation may modulate CCR5 expression, in agreement with the conclusions of de Roda Husman et al. [3]. Rather, our conclusion was that, in the patients we studied, “cell activation as evaluated by HLA-DR expression is not involved in the correlation between CCR5 density and virus load” (page 930). Nonetheless, in conflict with de Roda Husman et al. [3], we found that CCR5 expression, as measured by CCR5 density and by the frequency of CCR5<sup>+</sup>

cells in the whole (DR<sup>-</sup>) CD4<sup>+</sup> T cell population (figure 1) or in DR<sup>-</sup>CD4<sup>+</sup> T cells (data not shown), is not linked with the stage of HIV-1 infection. This discrepancy may be due to our intentional selection of asymptomatic patients, whose CD4 counts were >200 cells/ $\mu$ L, for our study, whereas the patients studied by de Roda Husman et al. were eventually symptomatic. Because we were aware of the influence of cellular activation on CCR5 expression, we wanted to avoid any inflammatory state linked to opportunistic infections that could have modified this expression and disturbed the link between CCR5 density and virus load. Alternatively, or additionally, the reason for the difference between the results of de Roda Husman et al. and ours may be technical. CCR5 expression alters with time at the surface of CD4<sup>+</sup> T cells in whole blood left at room temperature after being drawn, and, for this reason, we processed blood samples within 1 hour, whereas de Roda Husman et al. used cryopreserved blood cells. Variations in the delay before freezing, and freezing per se, could affect the results of CCR5 phenotyping.

Marmor et al. point to patient 1, in figure 1A in our article, showing the correlation between DR<sup>-</sup>CD4<sup>+</sup> T cell surface CCR5 density and HIV-1 load, as an “outlier.” This patient presents with a discrepancy between a moderate DR<sup>-</sup>CD4<sup>+</sup> T cell surface CCR5 density (7214 CCR5 molecules per cell) and a high DR<sup>-</sup>CD4<sup>+</sup> T cell surface CCR5 density (21,713 CCR5 molecules per cell), which is poorly correlated with a moderate viremia (18,924 copies/mL). This combination may be an example of the disturbing effect of inflammation on CCR5 density on the whole CD4<sup>+</sup> T cell population but not on the DR<sup>-</sup>CD4<sup>+</sup> T cells, which would explain why we found the correlation between virus load and CCR5 density on DR<sup>-</sup>CD4<sup>+</sup> T cells to be higher than the correlation between virus load and CCR5 density on DR<sup>-</sup>CD4<sup>+</sup> T cells. Interestingly, this discrepancy was temporary: when we repeated the measure on the same patient 6 months later, we found 6904 and 6603 CCR5 molecules per cell on DR<sup>-</sup> and DR<sup>-</sup>CD4<sup>+</sup> T cells, respectively. Eliminating this type of patient would, of course, improve the correlation between CCR5 density on the whole CD4<sup>+</sup> T cell population and HIV-1 load.

The extrapolation of the regression curve to zero CCR5 density predicting zero virus load is an elegant observation, and we thank Marmor et al. for it.

Finally, it seems that we have not been clear enough in presenting figure 5 in our article, which shows the evolution of CCR5 density at the surface of CD4<sup>+</sup> T cells infected in vitro by HIV-1. The purpose of this experiment, which has been repeated with the same results, was not to study the effect of CD4<sup>+</sup> T cell activation on CCR5 expression—CCR5 expression is up-regulated, down-regulated, or unmodified, depending on the stimulus—but rather to determine whether in vitro HIV-1 infection induces an increase in CD4<sup>+</sup> T cell surface CCR5 density. For this purpose, we stimulated peripheral blood lymphocytes (PBL) with phytohemagglutinin (PHA) and interleu-

## Dissociation of Immunologic and Virologic Responses to Highly Active Antiretroviral Therapy

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**Objective:** Immunologic markers, levels of HIV DNA, and infectious HIV were compared in partial responders (PR) to HAART who had high plasma HIV RNA levels but stable or increasing levels of CD4<sup>+</sup> peripheral blood mononuclear cells (PBMC), and patients with complete failure (CF) who had very low or decreasing levels of CD4<sup>+</sup> PBMC and high plasma HIV RNA levels.

**Design and Methods:** CD4 and CD8 levels were monitored by flow cytometry.  $\beta_2$ -microglobulin ( $\beta_2$ M) and neopterin levels were measured by quantitative enzyme immunoassays. Plasma and PBMC from 11 PR and 13 CF were analyzed for infectious HIV levels in limiting dilution cultures. Polymerase chain reaction (PCR) assays were used to quantify cellular HIV DNA and plasma HIV RNA.

**Results:** In comparison with CF, PR had little or no CD4<sup>+</sup> cell loss, a substantial increase in CD8<sup>+</sup> cells, significantly fewer positive plasma HIV cultures ( $p = .03$ ), lower frequencies of infectious HIV in total PBMC ( $p = .005$ ) and in CD4<sup>+</sup> PBMC ( $p < .001$ ), and lower frequencies of HIV DNA in CD4<sup>+</sup> PBMC ( $p = .007$ ).

**Conclusions:** Lower levels of infectious HIV and a lower frequency of CD4<sup>+</sup> PBMC that contain "productive" HIV DNA in PR as compared with CF may contribute to the stable or increasing CD4<sup>+</sup> PBMC levels of the PR. However, HAART may also have effects on lymphocyte homeostasis independent of its antiviral activity.

**Key Words:** HAART—Immunologic response—Virologic response—HIV.

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Highly active antiretroviral therapy (HAART), which includes at least one protease inhibitor (PI) with two reverse-transcriptase inhibitors (RTI), has significantly reduced morbidity and mortality rates in HIV-positive patients (1,2). Many patients experience both immunologic and virologic responses to HAART in terms of increased levels of CD4<sup>+</sup> peripheral blood mononuclear cells (PBMCs) and reduced levels of plasma HIV RNA,

respectively (3–5). Between 7% and 15% of HAART patients, however, have a seemingly paradoxical response to HAART in that their CD4<sup>+</sup> PBMC levels increase substantially but their levels of plasma HIV RNA remain high (6–11). In this report, we classify these patients as partial responders (PR) to HAART and contrast them with those with complete failure (CF) who have neither a significant rise in CD4<sup>+</sup> lymphocytes nor a fall in their plasma HIV RNA levels. Plasma HIV RNA levels are a strong predictor of progression to AIDS (12,13) and it has been suggested that these paradoxical responses are transient and may be explained by the rate of disease progression before treatment (10). The levels of infectious HIV and of HIV DNA in these patients with para-

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doxic responses, however, have not been previously analyzed. The following studies were undertaken to investigate the factors that may contribute to the dissociation of immunologic and virologic responses to HAART.

## METHODS

### Study Subjects

Flow cytometric quantification of PBMC was performed as described (14). The PR all had CD4<sup>+</sup> PBMC counts >135 cells/ $\mu$ l and included 7 individuals with stable CD4<sup>+</sup> PBMC counts (<20 cells/ $\mu$ l) and 4 others with increasing CD4<sup>+</sup> PBMC counts (>50 cells/ $\mu$ l) during an average follow-up time of  $9 \pm 3$  months. The CF included 9 people with persistently low CD4<sup>+</sup> PBMC counts (<60 cells/ $\mu$ l) and 4 others with decreasing CD4<sup>+</sup> PBMC counts (>85 cells/ $\mu$ l) during an average follow-up time of  $8 \pm 3$  months. HAART consisted of combinations of PIs including zidovudine, lamivudine, stavudine, didanosine, nevirapine, and delavirdine.

### Plasma Neopterin, $\beta_2$ -Microglobulin, and HIV RNA Quantification

Enzyme immunoassays were used to quantify plasma concentrations of neopterin (ICN, Costa Mesa, CA, U.S.A.) and  $\beta_2$ M (R&D Systems, Minneapolis, MN, U.S.A.). Plasma HIV RNA concentrations were determined using the Amplicor HIV-1 Monitor test (15) (Roche Diagnostic Systems, Branchburg, NJ, U.S.A.) or by bDNA assays (16) (Chiron, Emeryville, CA, U.S.A.).

### Quantification of HIV DNA and Infectious HIV

For quantification of HIV DNA, PBMC were purified from whole blood using Lymphocyte Separation Medium (ICN). PBMC were then lysed in a buffer containing proteinase K and the DNA quantified using a Hoechst dye. Lysates were coamplified for 30 cycles with an internal DNA quantification standard in a prototype assay that uses Amplicor HIV-1 Monitor v1.5 primers SK145-SKCC1B (17,18). Amplified products were quantified in microwell plates using the Amplicor HIV-1 Monitor format. This assay has been shown to yield highly reproducible results and HIV DNA levels determined by this method have been shown to be significantly correlated with plasma HIV RNA levels (19). Infectious units (IU) of HIV in plasma or PBMC were quantified by limiting dilution cultures as described (20,21). Briefly, fivefold dilutions of plasma or PBMC were cultured for 21 days with phytohemagglutinin (PHA)-stimulated PBMC from HIV-seronegative blood bank donors in the presence of T Cell Growth Factor (Cellular Products, Inc., Buffalo, NY, U.S.A.). At the end of 21 days, culture supernatants were analyzed for HIV p24 antigen by enzyme immunoassay (SAIC, Frederick, MD, U.S.A.). Supernatants were scored as either positive (>250 pg/ml) or negative for HIV p24 antigen and the tissue culture infectious dose 50% endpoint (TCID<sub>50</sub>) was calculated as previously described (22). The IU per ml of plasma or per million total PBMC (IUPM) are expressed as the reciprocal of the TCID<sub>50</sub>. The IUPM CD4<sup>+</sup> PBMC

were calculated by dividing the reciprocal of the TCID<sub>50</sub> by the percentage of the patient's PBMC expressing CD4.

### Statistical Analysis

Levels of PBMC and soluble markers of immune activation are reported as the arithmetic means (X)  $\pm$  standard error of the mean (SEM). Levels of HIV RNA, HIV DNA, and infectious HIV are reported as geometric means (X)  $\pm$  SEM. Statistical comparisons of PR and CF were performed using Student's, Mann-Whitney, Fisher's exact, or Spearman's tests.

## RESULTS

Antiretroviral drug use and levels of CD4<sup>+</sup> PBMC and HIV RNA in plasma of one PR are shown in Figure 1. After initiating HAART, this patient had a dramatic increase in CD4<sup>+</sup> PBMC counts from <20 cells/ $\mu$ l to >250 cells/ $\mu$ l over a period of 14 months. During this period, his CD8<sup>+</sup> PBMC count decreased from 1007 to 847 cells/ $\mu$ l. Except for one brief period after switching therapy to didanosine, nevirapine, and delavirdine, his plasma HIV RNA concentrations remained between 54,300 and 562,200 copies/ml. After again switching HAART drugs to high doses (3600 mg daily) of saquinavir soft gel capsules, low doses of ritonavir (800 mg daily) plus 80 mg/day stavudine (genotyping showed no mutation at position 75 of the pol gene), a dramatic decrease occurred in plasma HIV RNA levels to 1203 copies/ml and an additional increase in CD4<sup>+</sup> PBMC count to 360 cells/ $\mu$ l. In samples taken before this change of therapy when plasma HIV RNA levels were 181,300 copies/ml, we were unable to culture virus from his plasma and found low levels of infectious cell-associated HIV (16.2 IU per million PBMC and 75 IU per million CD4<sup>+</sup> PBMC). The levels of this patient's HIV DNA at this time were 5,893 per million total PBMC and 27,408 copies per million CD4<sup>+</sup> PBMC. Given that levels of plasma HIV RNA and CD4<sup>+</sup> PBMC are usually inversely related, the high levels of HIV RNA and stable or increasing CD4<sup>+</sup> PBMC levels in this patient prompted us to compare additional PR with complete failures who demonstrated no significant virologic or immunologic responses to HAART.

In PR ( $n = 11$ ) and CF ( $n = 13$ ) at the beginning of the follow-up period, no significant differences were found in the level of CD4<sup>+</sup> (PR =  $189 \pm 56$ ; CF =  $88 \pm 34$ ) or CD8<sup>+</sup> (PR =  $1026 \pm 183$ ; CF =  $866 \pm 178$ ) PBMC. Table 1 shows that the mean CD4<sup>+</sup> PBMC levels at the end of the follow-up period were significantly higher ( $p < .001$ ) in PR (258 cells/ $\mu$ l) than in CF (45 cells/ $\mu$ l). The PR showed a significant gain in CD4<sup>+</sup> PBMC ( $\Delta$ CD4 = +69) in contrast to CF who lost an average of 43 CD4<sup>+</sup> PBMC during the follow-up period

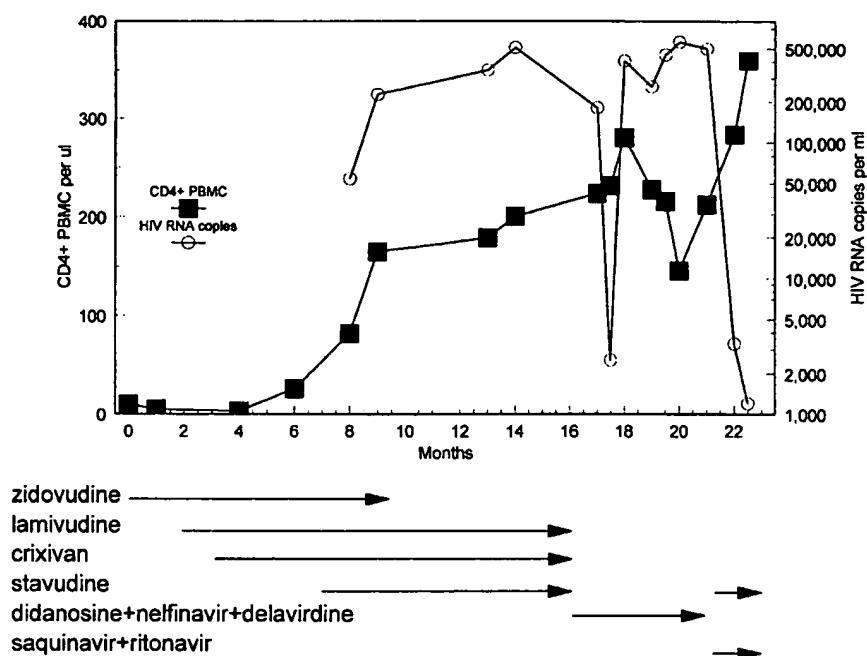


FIG. 1. Plasma HIV RNA, CD4<sup>+</sup> peripheral blood mononuclear cells, and antiretroviral drug usage in a partial responder.

of 8 to 9 months ( $p < .001$ ). Levels of CD8<sup>+</sup> PBMC were also significantly higher in PR than in CF ( $n < .001$ ). The change in CD8<sup>+</sup> PBMC levels ( $\Delta$ CD8) was also significantly different between PR and CF, increasing by an average of 274 CD8<sup>+</sup> PBMC/ $\mu$ l in PR and decreasing by an average of 309 CD8<sup>+</sup> PBMC/ $\mu$ l in CF ( $n < .001$ ) over 8 to 9 months of follow-up. Plasma markers of immune

activation,  $\beta_2$ M, and neopterin were measured in PR and CF only at the end of the follow-up period and were high in both groups but were not significantly different (Table 1).

Plasma and PBMC were collected at the end of the follow-up period and analyzed quantitatively for HIV RNA, HIV DNA, or IU of HIV (Table 1). The levels of

TABLE 1. Levels of CD4<sup>+</sup> and CD8<sup>+</sup> peripheral blood mononuclear cells (PBMC) plasma immune activation markers, and HIV in partial responders and complete failures to highly active antiretroviral therapy (HAART)

	Partial responders	Complete failures	Significance ( $p$ value)
	( $n = 11$ )	( $n = 13$ )	
CD4 <sup>+</sup> PBMC count (cells/ $\mu$ l) <sup>a</sup>	258 $\pm$ 47	45 $\pm$ 17	<.001
$\Delta$ CD4 <sup>+</sup> PBMC count (cells/ $\mu$ l) <sup>b</sup>	+69 $\pm$ 23	-43 $\pm$ 19	<.001
CD8 <sup>+</sup> PBMC count (cells/ $\mu$ l) <sup>a</sup>	1,300 $\pm$ 157	556 $\pm$ 91	<.001
$\Delta$ CD8 <sup>+</sup> PBMC count (cells/ $\mu$ l) <sup>b</sup>	+274 $\pm$ 150	-309 $\pm$ 116	<.001
$\beta_2$ -Microglobulin ( $\mu$ g/ml) <sup>a</sup>	6.8 $\pm$ 0.4	7.2 $\pm$ 0.1	NS
Neopterin (ng/ml) <sup>a</sup>	13.5 $\pm$ 2.4	13.9 $\pm$ 1.3	NS
Plasma HIV RNA/copies/ml <sup>a</sup> ( $\log_{10}$ $\pm$ standard error of the mean (SEM))	101,215 (5.0 $\pm$ 0.1)	192,581 (5.3 $\pm$ 0.1)	.045
HIV DNA copies/10 <sup>6</sup> PBMC <sup>a</sup> ( $\log_{10}$ $\pm$ SEM)	4,243 (3.6 $\pm$ 0.1)	4,634 (3.7 $\pm$ 0.2)	NS
HIV DNA copies/10 <sup>6</sup> CD4 <sup>+</sup> PBMC <sup>a</sup> ( $\log_{10}$ $\pm$ SEM)	26,914 (4.4 $\pm$ 0.1)	144,160 (5.2 $\pm$ 0.2)	.007
HIV DNA copies/ml <sup>a</sup> ( $\log_{10}$ )	6,944 (3.8)	6,487 (3.8)	NS
Infectious units HIV/ml plasma <sup>a</sup> ( $\log_{10}$ $\pm$ SEM)	3 (0.1 $\pm$ 0.3)	16 (1.2 $\pm$ 0.3)	NS
Infectious HIV units/10 <sup>6</sup> PBMC <sup>a</sup> ( $\log_{10}$ $\pm$ SEM)	7 (0.8 $\pm$ 0.2)	70 (1.8 $\pm$ 0.3)	.005
Infectious HIV units/10 <sup>6</sup> CD4 <sup>+</sup> PBMC <sup>a</sup> ( $\log_{10}$ $\pm$ SEM)	42 (1.6 $\pm$ 0.2)	2,165 (3.3 $\pm$ 0.3)	<.001
CD4 <sup>+</sup> IU/ml <sup>a</sup> ( $\log_{10}$ )	10.8 (1.0)	97.4 (2.0)	<.001

<sup>a</sup> At end of follow-up.

<sup>b</sup> Changes during follow-up.

NS, not significant ( $p > .05$ ). PBMC,  $\beta_2$ -microglobulin, and neopterin levels are reported by arithmetic means  $\pm$  SEM. HIV levels are reported as geometric means and as  $\log_{10}$   $\pm$  SEM. Levels of CD4<sup>+</sup> and CD8<sup>+</sup> peripheral blood leukocytes, plasma immune activation markers, HIV RNA, proviral HIV DNA, and infectious units of HIV were quantified as described in Methods.

plasma HIV RNA were only slightly lower ( $0.3 \log_{10}$ ) in PR as compared to CF ( $p = .045$ ). Both groups also showed substantial levels of HIV DNA in their PBMC but the mean levels were not significantly different. Because the CF had much lower levels of CD4<sup>+</sup> PBMC than PR the levels of HIV DNA per million CD4<sup>+</sup> PBMC were significantly higher ( $p = .007$ ) in the CF than in the PR (Fig. 2). The PR and CF both had high plasma HIV RNA levels but only a very small fraction of this HIV RNA represented HIV particles that were capable of replicating in tissue culture. Although the mean levels of IU of HIV in plasma were not significantly different between PR (3 IU/ml) and CF (16 IU/ml), HIV could be cultured from only 3 (27%) of 11 PR in comparison to 9 (69%) of 13 CF ( $p = .03$ ) (Fig. 3). The ratio of IU to HIV RNA in plasma was 1:12,036 in CF and 1:33,378 in PR, similar to reports in untreated individuals (23,24). When results of CF and PR were combined, the HIV IU/ml of plasma showed a significant correlation with the  $\log_{10}$  HIV RNA copy number in plasma ( $r = 0.625$ ;  $p < .001$ ).

Figure 4 shows that the PR had significantly lower levels than CF of IUPM HIV per million total PBMC mononuclear cells (7 versus 70;  $p = .005$ ). When results of CF and PR were combined, the IUPM and IU/ $10^6$  CD4<sup>+</sup> PBMC both showed significant correlations with the  $\log_{10}$  HIV RNA copy level in plasma ( $r = 0.4$ ;  $p < .05$ ). In that the PR had much higher levels than CF of CD4<sup>+</sup> PBMC, the mean levels of IUPM CD4<sup>+</sup> PBMC was more than 50-fold lower in PR than CF (42 versus 2,165;  $p < .001$ ). When corrected for CD4 count, the PR had about 10-fold fewer IU per ml of blood than CF (10.8 versus 97.4, respectively).

## DISCUSSION

These results demonstrate that, despite a similar number of HIV-infected cells per ml, CF have both a higher proportion of HIV-infected CD4<sup>+</sup> cells and a higher proportion of those that are able to initiate new virus production in vitro. The observed differences between PR and CF in total viral RNA and "infectious" RNA per ml were consistent with this finding but smaller than might be expected, given a 10-fold difference in "productively infected" CD4<sup>+</sup> cells per ml.

Because PI are thought to function primarily by rendering newly produced virus noninfectious (25), this decreased infectivity in PR versus CF may be due to a limited virologic response in HAART that was not evident in the measurement of viral RNA alone. It has been suggested that PI may act at more than one step in retroviral replication (26) and differential effects of PI and/or RTI in PR and CF may also contribute to these differences in infectivity. Faye et al. have reported data consistent with our observations and suggest decreased viral fitness is associated with a gain in CD4<sup>+</sup> PBMC in patients with discordant CD4 and plasma HIV RNA responses to PI therapy (27).

Although observed differences in infectivity may contribute to the improved immunologic responses seen in PR, the critical question is: are these differences large enough to account for the degree of stability and/or increase in CD4 counts in the PR group? We observed a 10-fold difference in IUPM PBMC between PR and CF (7 versus 70 IU per million). A recent study has evaluated the relationship between cell-associated infectious HIV-1 and progression to AIDS or death in untreated individuals (28). Although differences in culture condi-

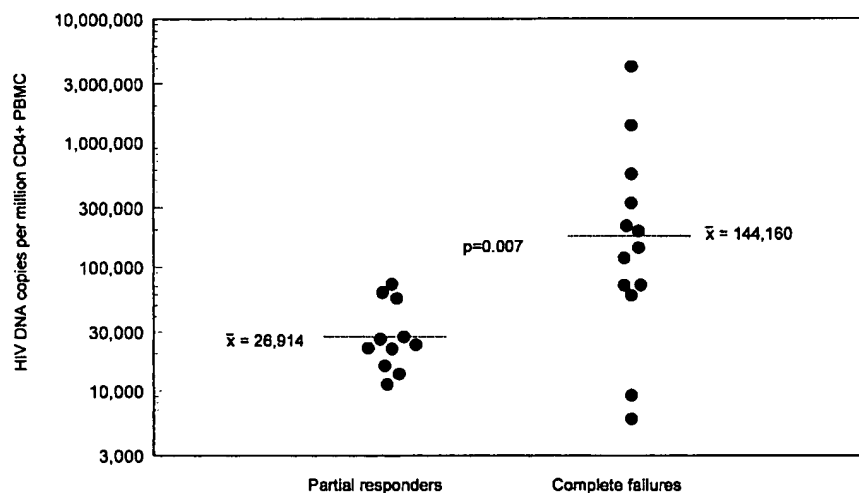


FIG. 2. HIV DNA Levels in CD4<sup>+</sup> peripheral blood mononuclear cells from partial responders and complete failures.



whether either or both of these mechanisms may be occurring in PR.

A recent study suggested that the previous rate of CD4 depletion and long-term viral load reduction played an important role in discordant immunologic and virologic responses in the first few months after initiation of HAART (10). Levels of infectious HIV and HIV DNA and their influence on immunologic and virologic responses to HAART were not, however, evaluated in that study. Although data regarding CD4<sup>+</sup> depletion rates before initiation of HAART are not available for our study subjects, the duration of the discordant responses for more than 1 year in some of the PR make this an unlikely explanation for our observations.

Because peripheral blood represents only  $\leq 2\%$  of the total lymphocyte pool, even small effects of PI and/or RTI on lymphocytes in lymph nodes may influence our observations of PBMC in PR. It has been suggested that increases in peripheral blood shortly after the initiation of HAART may be primarily the result of lymphocyte redistribution from the lymph nodes to peripheral blood (31). Other recent studies (34–37) show that HAART reverses some of the immunoactivation that is associated with HIV infection and that this may be independent of its antiviral activity (36). This possibility offers an appealing explanation for the findings reported here.

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# Human Megakaryocytes Have a CD4 Molecule Capable of Binding Human Immunodeficiency Virus-1

By Yamil H. Kouri, William Borkowsky, Michael Nardi, Simon Karpatkin, and Ross S. Basch

Most human megakaryocytes (MGKs) express the CD4 antigen on their surface. Approximately 25% have a CD4 receptor density comparable to that of CD4<sup>+</sup> T cells (Basch et al, *Proc Natl Acad Sci USA* 87:8085, 1990). In these studies, we show: (1) the presence of mRNA for CD4 in human MGKs; (2) the binding of human immunodeficiency virus-1 (HIV-1) to human MGKs; (3) the inhibition of binding by anti-CD4 (Leu3a) antibody or rCD4; (4) the infection of a human MGK line, CHRF-288 with HIV-1; and (5) inhibition of infection with anti-CD4. Human MGKs have mRNA for CD4 as shown by in situ hybridization with an RNA probe synthesized from a 3-kb cDNA sequence of plasmid pSP65.T4.8 containing the full-length CD4 sequence.

**T**HROMBOCYTOPENIA is a frequent complication of human immunodeficiency virus type-1 (HIV-1) infection<sup>1</sup> and appears to result from a combination of decreased platelet survival<sup>2,4</sup> and impaired platelet production.<sup>2,6</sup> The platelet count generally improves promptly after reticulo-endothelial cell suppression or ablation, suggesting an immunologic etiology, or within 1 week after anti-HIV-1 treatment with azidothymidine (AZT),<sup>5,6</sup> suggesting megakaryocyte suppression, possibly due to the invasion of megakaryocytes (MGKs) with HIV-1. The evidence that MGK can be directly infected by HIV-1 is supported by morphologic abnormalities of megakaryocytes,<sup>7</sup> the detection of HIV-1 viral mRNA in some MGKs of HIV-seropositive individuals,<sup>8</sup> the susceptibility of MGK cell lines in culture to productive infection with HIV-1,<sup>9</sup> decreased MGK precursors,<sup>10</sup> and improvement of impaired platelet turnover after treatment with AZT, whereas decreased platelet survival remains unchanged.<sup>4</sup> However, a mode of entry for the HIV-1 virus has not been established and HIV-1 viral antigens have not been detected on human MGK isolated from HIV-1-infected patients.

We have shown that normal human MGKs express the CD4 antigen on their surface.<sup>11</sup> This antigen is the high-affinity receptor for the envelope gp120 protein<sup>12-14</sup> of HIV-1

MGKs (23%  $\pm$  17%) bound HIV-1, as determined by anti-gp120 and anti-CD41 staining. Binding to human MGKs could be inhibited 55% to 75% with anti-CD4 or rCD4, respectively. Infection of a CD4<sup>+</sup> MGK line (CHRF-288) could be accomplished with HIV-1, as determined by proviral DNA polymerase chain reaction and p24 production. Preincubation with anti-CD4 inhibited apparent proviral DNA infection by 100% and p24 production by 65% to 70%. Thus, human MGKs have a CD4 receptor capable of binding HIV-1. Using this receptor, HIV-1 can infect cells representative of the MGK lineage.

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and, if expressed as an integral part of the cell surface of MGKs, it could provide a portal of entry for the virus. However, the presence of the previously reported antigen could represent a nonfunctioning CD4 molecule, a cross-reacting epitope, or passive adsorption of soluble antigen onto the cell surface rather than synthesis and insertion of a functional receptor on the MGK membrane surface. Such adsorption phenomena have been reported for the T-cell antigen CD8 on thymocytes<sup>15</sup> and for HLA antigens on platelets.<sup>16</sup> In this study, we provide evidence for a functional CD4 receptor on human MGKs by showing: (1) the presence of CD4 mRNA in human MGKs; (2) the binding of HIV-1 to normal human MGKs; (3) inhibition of that binding by antibody to CD4 and soluble rCD4; (4) infection of a MGK line (CHRF-288) with HIV-1; and (5) inhibition of infection with anti-CD4.

## MATERIALS AND METHODS

Normal human bone marrow specimens were obtained from discarded orthopedic surgical specimens (hip surgery) and were resuspended in CATCH medium.<sup>11</sup> The tissue was gently pipetted with a 12-mL plastic syringe through an 18-gauge needle until a single cell suspension was obtained. The samples were then underlaid with an equal volume of Ficoll-Hypaque (1.077 g/mL; Sigma, St Louis, MO) and centrifuged at 2,000g for 20 minutes at room temperature. The cells on the interface were collected, washed, resuspended in CATCH, underlaid with an equal volume of 38% isosmotic Percoll (Pharmacia, Piscataway, NJ) in CATCH medium, and centrifuged at 2,000g at 4°C for 20 minutes. The cells at the interface were again isolated, washed, and used for in situ hybridization and binding experiments (see below).

The CHRF-288 MGK line was kindly supplied by Dr M. Lieberman (University of Cincinnati Medical Center, Cincinnati, OH). This cell line was obtained from a patient with megakaryoblastic leukemia.<sup>17</sup> These cells are unique in that they have characteristic markers for MGKs and platelets, yet do not express the erythroid markers glycophorin A and hemoglobin, the myeloid marker myeloperoxidase, or markers for T and B cells.<sup>17</sup>

## Synthesis of Radiolabeled Probe for CD4 mRNA

The plasmid pSP65.T4.8 was obtained from Dr D. Littman (University of California, San Francisco, CA). It contains a 3-kb sequence that encodes the entire T4 (CD4) cDNA in the antisense orientation. An RNA probe was synthesized by linearizing the plasmid with *Xba*I (Boehringer Mannheim, Indianapolis, IN) and incubating it with SP6 RNA polymerase in the presence of transcription buffer, 10 mmol/L dithiothreitol (DTT), ribonuclease inhibitor, 0.5 mmol/L

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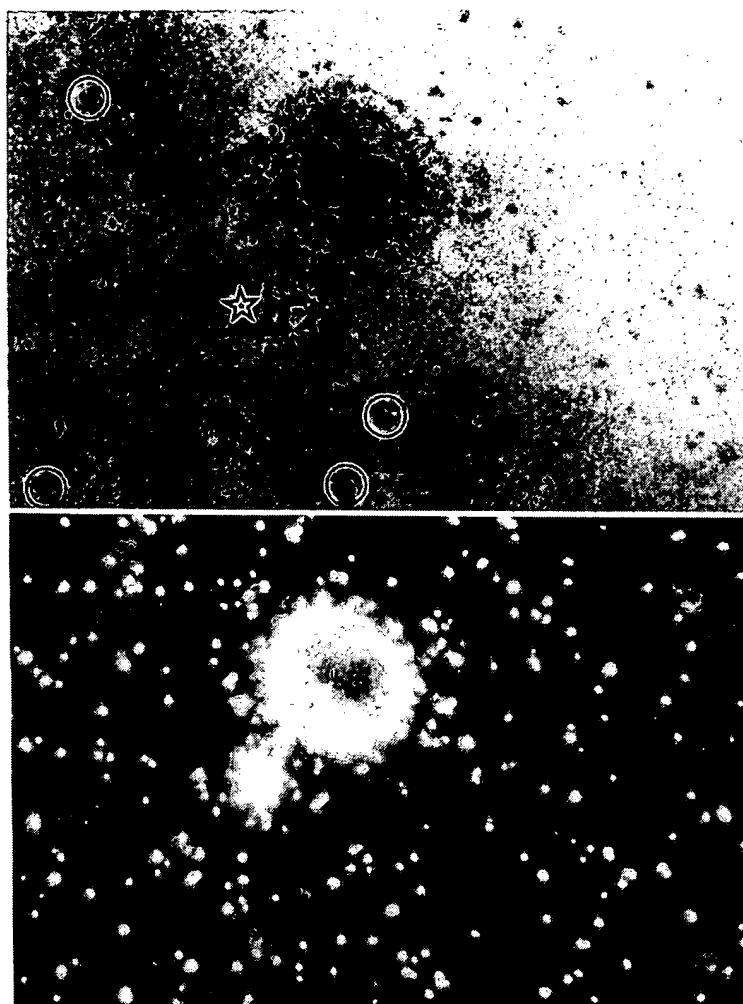
Presented at the Annual Meeting of the American Society of Hematology, Denver, CO, December 9, 1991 (Blood 78:361a, 1991 [abstr, suppl 1]).

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**Fig 1.** In situ hybridization of CD4 mRNA expression by a megakaryocyte. (Top) A photomicrograph of a bright field image of a cytocentrifuged preparation of low-density human bone marrow cells isolated from a Percoll gradient and hybridized with a  $^{35}\text{S}$ -labeled antisense RNA probe specific for CD4. The large cell in the center is an MGK. The small, densely labeled cell (marked with a star) is a lymphocyte. The circles indicate four cells that did not hybridize with the probe. The grains appear to be distributed around the labeled cells because of optical sectioning; grains lying directly over the cells are not in focus. (Bottom) A dark field image of the same area. The intense labeling of the MGK and the starred lymphocyte is readily apparent (original magnification  $\times 630$ ).

of each unlabeled nucleotide (adenosine triphosphate [ATP], guanosine triphosphate [GTP], and uridine triphosphate [UTP]), and 10  $\mu\text{L}$  of [ $^{35}\text{S}$ ] cytidine triphosphate (CTP) (NEN/DuPont, Wilmington, DE). The DNA template was digested with DNase I (RQ1, Rnase free) in the presence of ribonuclease inhibitor and 1 mg/mL of carrier RNA. After removal of enzyme proteins by extraction with phenol/chloroform, the solution was made 2 mol/L ammonium acetate and the riboprobe precipitated with 100% ethanol in dry ice. The pellet was washed twice with 70% ethanol, dried, resuspended in 5  $\mu\text{L}$  of 50 mmol/L DTT, and stored at  $-20^\circ\text{C}$  until use. The probes were used within 2 days. Between  $10^6$  and  $2 \times 10^7$  cpm were incorporated into each probe. All reagents were purchased from Promega (Madison, WI) and incubations were performed at  $37^\circ\text{C}$ .

The plasmid P.I.L-3 GEM, encoding a fragment of murine interleukin-3 (IL-3), was used as a negative control. It was provided by Dr S. Gillis of Immunex Corp (Seattle, WA).

#### *In Situ Hybridization*

Bone marrow cells were resuspended in CATCH media at a concentration of  $2$  to  $3 \times 10^7/\text{mL}$  and applied on an area of 20  $\text{mm}^2$  over a slide coated with 3-aminopropyltriethoxysilane (Digene, Silver Spring, MD). After incubation in a humidified chamber at room temperature for 30 minutes, the slides were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 minutes. In situ

hybridization was then performed as previously described.<sup>18</sup> Diethylpyrocarbonate (DEPC) was included in all of the buffers and was also present in the water used to wash the glassware required for these experiments. In selected experiments, immunocytochemistry was performed for MGK glycoprotein (GP) IIIa (see below). Kodak NTB-2 emulsion (Eastman Kodak, Rochester, NY) was used to coat the slides for autoradiography. The slides were exposed from 5 to 21 days at  $4^\circ\text{C}$  and were counterstained with Giemsa.

#### *Proviral DNA Assay for HIV-1*

CHRF-288 cells were divided into 1-mL aliquots of  $1 \times 10^6$  cells/mL. Ten microliters of Leu3a (Becton Dickinson, San Jose, CA) was added to 1 aliquot and 10  $\mu\text{L}$  of media was added to another. Both aliquots were incubated for 15 minutes at room temperature. The cells were then washed three times with Hanks' Balanced Salt Solution. One milliliter of supernatant from an HIV-1<sub>IIIb</sub>-infected cell line (HBP-9) that was filtered through a 0.22  $\mu\text{m}$  filter was added to each aliquot for 30 minutes. The cells were then washed three times and placed at  $37^\circ\text{C}$  overnight. The next day the cells were washed five times and centrifuged to a pellet for processing for the polymerase chain reaction (PCR). Another aliquot of CHRF-288 cells was washed and pelleted and used for a negative control.

The cell pellet was lysed by adding equal volumes of a solution containing TRIS (10 mmol/L, pH 8.3), KCl (100 mmol/L), and

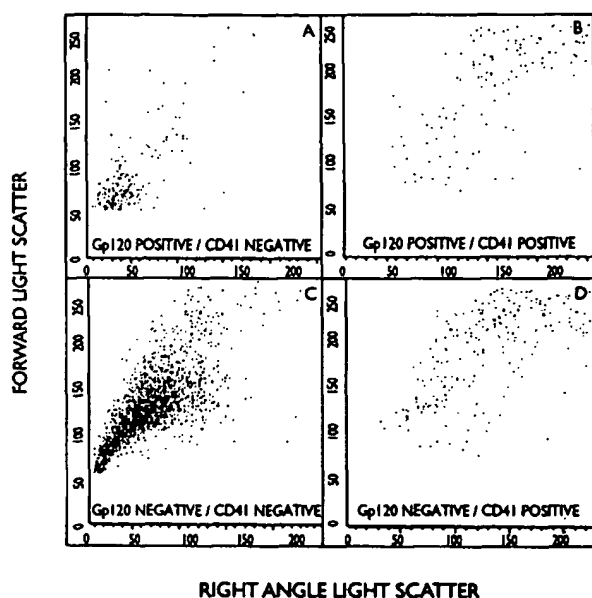


Fig 2. Light scatter analysis of the various cell populations after staining with anti-gp120-PE and anti-CD41 (GPIIb-GPIIIa)-FITC. (A) Cells that bind HIV-1 (gp120-PE), but are not stained by CD41-FITC. This light scatter pattern is characteristic of lymphocytes and monocytes. (B) Cells that bind HIV-1 and stain with CD41-FITC. This light scatter pattern is characteristic of MGKs. (C) Double-negative cells. (D) Cells that do not bind HIV-1 and stain with CD41-FITC. This pattern is characteristic of MGKs.

MgCl<sub>2</sub> (2.5 mmol/L) to a solution containing TRIS (10 mmol/L, pH 8.3), 1% Tween 20, 1% NP40, and proteinase K (200 µg/mL) to yield a final cell concentration of  $1.5 \times 10^6$  cells/mL. The suspension was incubated at 56°C for 2 hours and then at 95°C for 15 minutes. Ten-microliter aliquots were added to 90-µL aliquots of PCR reaction buffer containing the primer pair (see below) at concentrations suggested by the manufacturer (Cetus/Perkin-Elmer, Norwalk, CT), with a final magnesium concentration of 2.5 mmol/L. In addition, bovine serum albumin was included in the reaction mixture at a concentration of 50 µg/mL. The mixture was subjected to 1 cycle of heating to 95°C for 2 minutes, followed by incubation at 80°C for 8 minutes. It was then followed by 30 cycles of annealing performed at 53°C (2 minutes), TAQ polymerase-mediated chain extension at 72°C (1 minute), and denaturation at 94°C (1 minute). A final annealing step followed by a prolonged extension step for 7 minutes completed the amplification.

The primer pair SK38/39, which encompasses the core region (gag 1551-1665), was used for sample amplification.<sup>19</sup> The mixture was tested for the presence of HIV-1 DNA using a liquid hybridization protocol. Thirty microliters of the mixture is hybridized with 10 µL of a cocktail containing 4 µL of 1.5 mol/L NaCl, a <sup>32</sup>P-end-labeled SK19 probe located at gag 1595-1635 (250,000 cpm/sample), and Tris-EDTA for at least 0.5 hours at 55°C and then electrophoresed through an 8% polyacrylamide gel and detected by autoradiography. The presence of HIV-1-DNA was associated with a displaced larger molecular weight band in the same area as the positive control of the probe alone. All assays included 1 positive and 2 negative controls. The DNA and reaction mixture preparation was routinely performed in separate rooms from where the hybridization assay was performed to reduce the risk of amplicon contamination.

#### HIV-1p24 Secretion

HBP-9 (CD4<sup>+</sup>) T cells or CHRF-288 MGK ( $1 \times 10^6$ ) were incubated with either 0 or 10 µg of Leu3a in 1 mL of RPMI at room

temperature for 30 minutes. The cells were washed, pelleted, and incubated with 0.5 mL of cell-free HIV-1<sub>IIIB</sub> for 30 minutes. Cells were rewashed, pelleted, and incubated with 1.1 mL of RPMI for 9 days at 37°C. Aliquots were removed and assayed for p24 with an enzyme-linked immunosorbent assay (ELISA) kit provided by Coulter (Hialeah, FL).

#### Antibodies

For immunocytochemical staining, we used the murine monoclonal antibody (MoAb) LK-6 (IgG1,  $\kappa$  IgG subclass), which was raised in our laboratory by Dr L.-X. Liu and has a high affinity for both native and denatured platelet/GMK GPIIIa. For binding and blocking experiments, we used a fluorescein-conjugated MoAb against the GPIIb/GPIIIa complex (CD41a, clone P2, mouse IgG<sub>1</sub>), purchased from AMAC (Westbrook, ME). Unconjugated anti-CD4 (Leu 3a, clone SK3, mouse IgG<sub>1</sub>) was purchased from Becton Dickinson. Sheep antisera directed against gp120 of HIV-1 (Lot DV-012) was provided by the National Institutes of Allergy and Infectious Diseases, AIDS Research and Reference Reagent Program. Mouse MoAb against HIV-1gp120 was purchased from Specialty Diagnostics (DuPont, Wilmington, DE). Affinity-purified rabbit antiserum Ig conjugated to biotin was purchased from Jackson ImmunoResearch (West Grove, PA). Streptavidin coupled to phycoerythrin (PE) was purchased from Becton Dickinson. PE-conjugated goat F(ab')<sub>2</sub> antimouse Ig (H&L) was purchased from Caltag (San Francisco, CA). Anti-CD8 (Leu 2a,

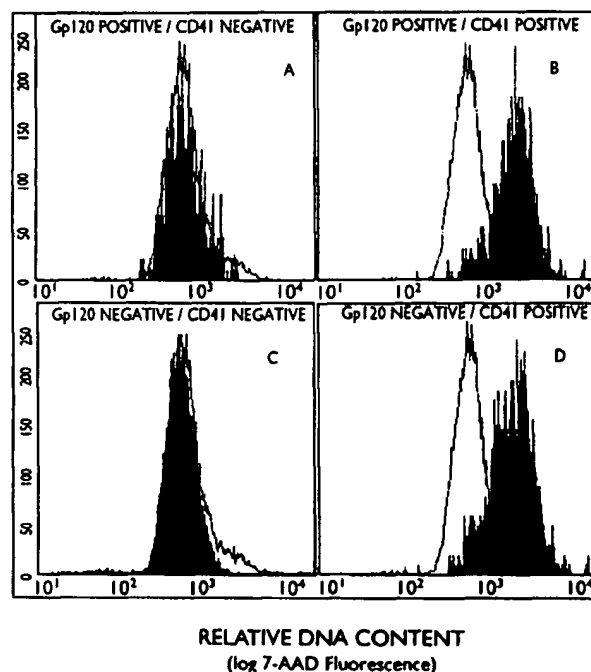


Fig 3. DNA distribution pattern of cells incubated with HIV-1 and stained with anti-gp120 and anti-CD41, based on staining with 7-AAD. The solid histogram represents the DNA content of the cells with the indicated serologic properties. The open histogram shows the distribution of DNA in the entire population isolated from the Percoll gradient. (A) Cells that bind HIV-1 gp120, but are negative for CD41. These cells are predominantly lymphocytes and monocytes. (B) Cells that bind HIV-1 gp120 and stain positively with CD41 (MGKs). The DNA content of the major population corresponds to a ploidy of 8 to 16N and suggests that MGKs and MGK precursors predominate in this fraction. (C) Double-negative cells. (D) Cells that stain for CD41 (MGKs), but do not bind HIV-1 gp120.

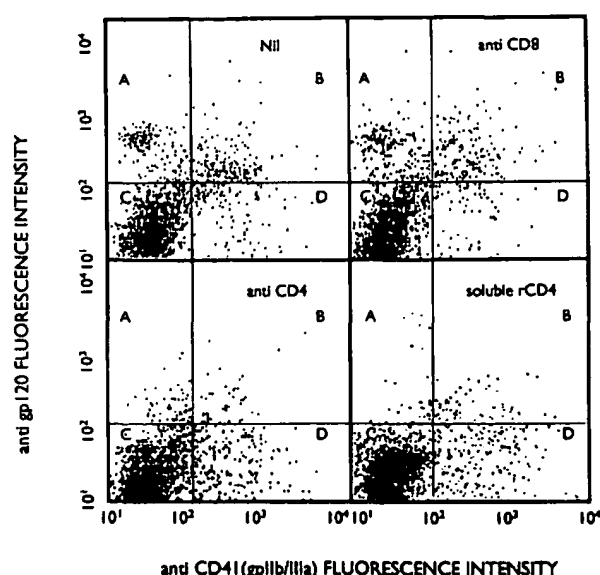


Fig 4. Representative two-color cytofluorogram of low-density human bone marrow cells incubated with HIV-1 and stained with anti-HIV-1 gp120-PE (ordinate) and anti-CD41-FITC (GPIIb-GPIIIa) (abscissa). Left upper panel, control binding experiment. Cells in quadrant A are stained with anti-gp120-PE, but not with CD41-FITC. Cells in quadrant B (MGKs) are positive for both gp120-PE and CD41-FITC. Cells in quadrant C are double negative. Cells in quadrant D (MGKs) are only stained with CD41-FITC. Right upper panel, effect of isotype-matched control anti-OKT8 Ab on HIV-1 binding. The pattern obtained here is indistinguishable from the control pattern in left upper panel A. Left lower panel, effect of anti-CD4 on HIV-1 binding. Treatment with Leu3a blocks binding of HIV-1 (staining with anti-gp120). Note the decrease in the proportion of cells in quadrants A and B and increase in quadrants C and D compared with that of the upper two panels. Right lower panel, effect of treatment with rCD4 on HIV-1 binding. Note that soluble rCD4 is as effective as anti-CD4 antibody in preventing the binding of HIV-1 to cells staining both positively and negatively for CD41 (compare quadrants B and A with D and C).

mouse IgG<sub>1</sub>) was purchased from Becton Dickinson for use as a control.

#### Immunocytochemistry

Immunocytochemical staining of GPIIb (a major platelet and MGK surface antigen) was performed in hybridization studies by incubating the slides in a moist chamber at room temperature with 10 µg/mL of Ab LK-6 in PBS for 45 minutes. The samples were washed and incubated with a 1:60 dilution of goat antimouse IgG (Fc domain) conjugated to alkaline phosphatase. Enzymatic activity

was detected by using an alkaline phosphatase detection kit with fast red violet (Sigma).

#### Immunofluorescence

Two-color immunofluorescence analysis was performed as described previously<sup>12</sup> using fluorescein (FL)-conjugated and PE-conjugated antibodies. Staining was performed at 4°C for approximately 30 minutes, unless otherwise noted.

#### DNA Staining

The DNA dye 7-aminoactinomycin D (7-AAD) was used in combination with two-color immunofluorescence to stain DNA.

#### Binding Experiments

Percoll-enriched bone marrow cells were resuspended in RPMI 1640 (GIBCO, Grand Island, NY) and mixed with equal volumes of supernatant from an HIV-1-infected H9 cell line grown in RPMI/10% fetal calf serum (FCS). The HIV-1 used was a patient isolate. One milliliter of supernatant contained a P24 antigen content of 20 to 50 ng/mL. Cells were incubated with HIV-1 at 4°C for 30 minutes, washed, resuspended at a concentration of approximately  $2 \times 10^7$ , and stained for 30 minutes with either mouse anti-gp120 or sheep anti-gp120. When mouse Ab was used, the secondary Ab was goat F(ab)<sub>2</sub> anti-MiGg conjugated to PE. When the primary Ab was from sheep, rabbit anti-sheep IgG coupled to biotin was used, followed by 15 minutes of incubation with streptavidin conjugated to PE. To block potentially unoccupied binding sites on the secondary Abs, all samples were resuspended in 10% normal mouse serum for 10 minutes before being stained with CD41 conjugated to FL. The specimens were then analyzed by flow cytometry.

For blocking experiments, the samples were preincubated at 4°C for 30 minutes with either Leu3a, Leu2a, rCD4 (0.1 to 10 µg/100 µL cell suspension), Leu2a (1 µg), or normal human Fab (1 µg, molecular weight  $\pm$  50 Kd), and then mixed with HIV-1-infected supernatant and stained as described above. Soluble rCD4 was obtained from the National Institutes of Allergy and Infectious Diseases, AIDS Research and Reference Reagent Program (Lot no. 55-89-ABT14).

#### Flow Cytometry

A Becton Dickinson FACScan cytometer equipped with a 15-mW argon laser emitting at 488 nm was used to quantify cell fluorescence. Viable cells were gated on the basis of both forward and 90° light scatter. FL emission was measured by filtering the light passed by the first dichroic mirror through a narrow band green filter (530  $\pm$  10 nm). PE and 7-AAD fluorescence were detected by sequential dichroic mirrors reflecting light of wavelengths longer than 640 nm. Electronic compensation was used to eliminate spectral overlap between FL and PE and between PE and 7-AAD. Ten thousand to 50,000 events were accumulated in list mode and later analyzed.

Table 1. Inhibition of Binding of HIV-1 to Low-Density Human Bone Marrow Cells by Anti-CD4 Antibodies and Soluble rCD4

Treatment* (n)	% Binding			% Inhibition of Binding		
	Total	CD41 <sup>-</sup>	CD41 <sup>+</sup>	Total	CD41 <sup>-</sup>	CD41 <sup>+</sup>
None (7)	9.6 $\pm$ 4.6	6.8 $\pm$ 3.8	2.8 $\pm$ 1.8	—	—	—
Leu 3a (4)	3.0 $\pm$ 1.5	1.1 $\pm$ 0.6	1.9 $\pm$ 1.1	69 $\pm$ 12	84 $\pm$ 11	32 $\pm$ 5.5
rCD4 (3)	1.6 $\pm$ 1.4	0.8 $\pm$ 1	0.8 $\pm$ 0.6	83 $\pm$ 22	88 $\pm$ 26	71 $\pm$ 7
OKT8 (1)	9.0	5.4	3.6	0	6	0
IgG F(ab) (1)	10.8	8.6	2.2	0	0	0

\* Inhibitors were used at 1 to 2 µg/100 µL cell suspension.

**Table 2. Dose-Response Inhibition of the Binding of HIV-1 by Anti-CD4 and Soluble rCD4**

Concentration*	% Inhibition		
	Total Population	CD41 <sup>+</sup> /gp120 <sup>+</sup>	CD41 <sup>+</sup> /gp120 <sup>-</sup>
(A) Leu 3a			
0 $\mu$ g	0	0	0
0.1 $\mu$ g	0	0	0
1.0 $\mu$ g	90	97	35
10.0 $\mu$ g	94	98	55
(B) rCD4			
0 $\mu$ g	0	0	0
0.1 $\mu$ g	0	0	25
1.0 $\mu$ g	95	97	75
10.0 $\mu$ g	74	79	34

\* Inhibitors were used per 100  $\mu$ L cell suspension.

## RESULTS

### *In Situ Hybridization*

As illustrated in Fig 1, MGKs express readily detectable levels of CD4 mRNA. A smaller mononuclear cell, presumably a T lymphocyte or monocyte, is also clearly positive. About 20% of morphologically identifiable MGKs (very large cells with prominent lobulated nuclei and abundant cytoplasm) were positive. When immunocytochemical staining was performed, both small (presumably early) and large size cells that were stained for the platelet/MGK surface antigen GPIIIa expressed CD4 mRNA. Platelets had no detectable CD4 mRNA.

### *Binding Experiments*

Low-density human bone marrow cells, isolated from a Percoll density gradient, contain  $14.4\% \pm 9.7\%$  MGKs (range, 4.9% to 34%). The MGK-enriched population was incubated with HIV-1 in the presence and absence of inhibitors of the binding of HIV-1gp120 to its CD4 receptor. In the absence of inhibition, HIV-1 bound to  $9.6\% \pm 4.6\%$  of cells (range, 3.0% to 18%).

**Light scatter pattern.** Approximately two-thirds of the HIV-1-binding cells ( $6.8\% \pm 3.8\%$  of the total; range, 0.8% to 12%) were lymphocytes and monocytes because their light scatter pattern was characteristic of small hypogranular cells and they were not stained with CD41 (Fig 2, left lower panel). Approximately one-third of the HIV-1-binding cells were MGKs, as judged by their light scatter pattern and positive reactivity with anti-CD41 (right upper and lower panels). Among MGKs (CD41<sup>+</sup> cells),  $23\% \pm 17\%$  bound HIV-1. This represents  $2.8\% \pm 1.8\%$  of the total low-density bone marrow sample (range, 0.9% to 6.4%).

**Relative DNA content.** Figure 3 shows the DNA content of CD41<sup>+</sup> cells (right upper and lower panel). Most of the CD41<sup>+</sup> cells are polyploid. The modal ploidy for MGKs was 16N. There was no significant difference between the MGKs that bound HIV-1 and those that did not.

**Effect of anti-CD4 and rCD4 on binding of HIV-1 to MGKs.** Figure 4 shows the ability of both anti-CD4 (Leu3a) and rCD4 to inhibit binding of HIV-1. MGK (CD41<sup>+</sup> cells) with bound HIV-1 are shown in quadrant B of all four panels. This binding is significantly inhibited by anti-CD4 (lower left

panel),  $32\% \pm 5.5\%$  at 1  $\mu$ g/100  $\mu$ L (Table 1), and 55% at 10  $\mu$ g/100  $\mu$ L (Table 2). Similar inhibition was produced with soluble rCD4 (lower right panel;  $71\% \pm 7\%$ , Table 1). Anti-CD8 (Leu2a), a control antibody of the same class as Leu3a, produced no inhibition of HIV-1 binding to low-density human bone marrow cells (upper right panel). In addition, no inhibition was noted with IgG F(ab), a protein of similar molecular weight as rCD4 (data not shown). The effect of these reagents on the binding of HIV-1 to CD41<sup>+</sup> cells is also illustrated in quadrant C of all four panels. The binding to small hypogranular cells (T cells and monocytes), not stained with CD41, is shown in quadrant A of all four panels. Incubation with anti-CD4 (lower left panel) inhibited  $84\% \pm 11\%$  of the HIV-1 binding (Table 1). Similar inhibition ( $88\% \pm 26\%$ ) was obtained with soluble rCD4 (lower right panel) (Table 1).

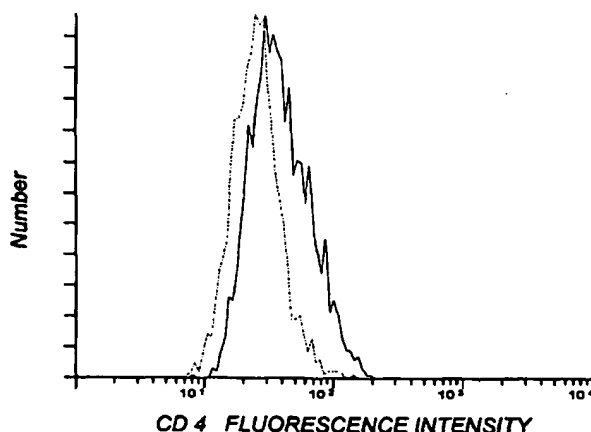
The inhibition of binding of HIV-1 by Leu3a and rCD4 was dose dependent, and maximal at 1 to 10  $\mu$ g/100  $\mu$ L (Table 2). Almost all of the HIV-1 binding to small hypogranular (CD41<sup>+</sup>/gp120<sup>-</sup>) cells could be blocked at 1  $\mu$ g/mL, but only 55% to 75% of the binding to MGKs could be blocked.

### *CD4-Dependent Infection of CHRF-288 MGKs*

**Fluorescence flow cytometry.** Figure 5 demonstrates the reactivity of Leu3a with CHRF-288 cells. CD4 appears to be present on most cells, but with relatively low intensity compared with CD4<sup>+</sup> T cells.

**Proviral DNA.** Liquid hybridization of the amplified HIV-1 product to a P<sup>32</sup>-labeled probe showed the presence of HIV-1 DNA in CHRF-288 cells incubated with cell-free HIV-1. Preincubation of CHRF-288 with Leu3a antibody before exposure to HIV-1 resulted in inhibition of the signal derived from the amplified core (gag) DNA (Fig 6). The infectivity of CHRF-288 cells is relatively low compared with that of HBP T cells.

**HIV-1p24 production by HIV-1<sub>IIIb</sub>-infected CHRF-288 MGKs.** Figure 7 shows infection and proliferation of HIV-1<sub>IIIb</sub> in CHRF-288 MGKs as designated by p24 antigen de-



**Fig 5.** Fluorescence histogram of CHRF-288 MGKs stained with anti-CD4 (Leu3a-FITC). (····) MGKs stained with irrelevant isotype-matched control mouse MoAb. (—) The staining of CHRF-288 with Leu3a.

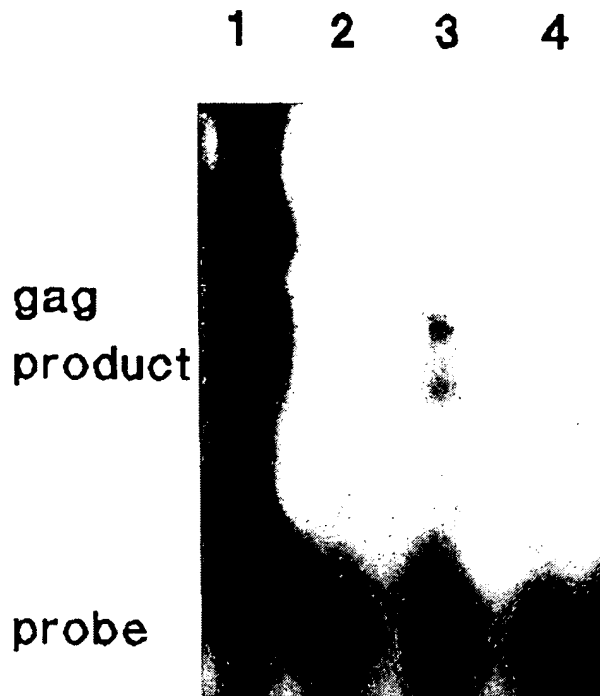


Fig 6. Proviral DNA in HIV-1-infected CHRF-288 MGKs. Liquid hybridization was performed using the PCR gag product obtained from primers SK38/39 and the P<sup>32</sup>-SK19 probe. Lanes 1 through 4 represent PCR probe and gel-retarded gag products of DNA extracted from cells in the absence and presence of Leu3a (anti-CD4 antibody). Lanes 1 and 2 contain DNA from H9 cells and lanes 3 and 4 contain DNA from CHRF-288 cells. Lanes 2 and 4 represent cells preincubated with Leu3a.

fection. Note the 65% to 70% inhibition of infection with Leu3a on days 9 and 7, respectively. CHRF-288 cells produced 13% of the p24 produced by HBP T cells.

#### DISCUSSION

We have previously shown that human MGKs have serologically detectable CD4 on their surface.<sup>11</sup> We now show that these cells express the mRNA that encodes the CD4

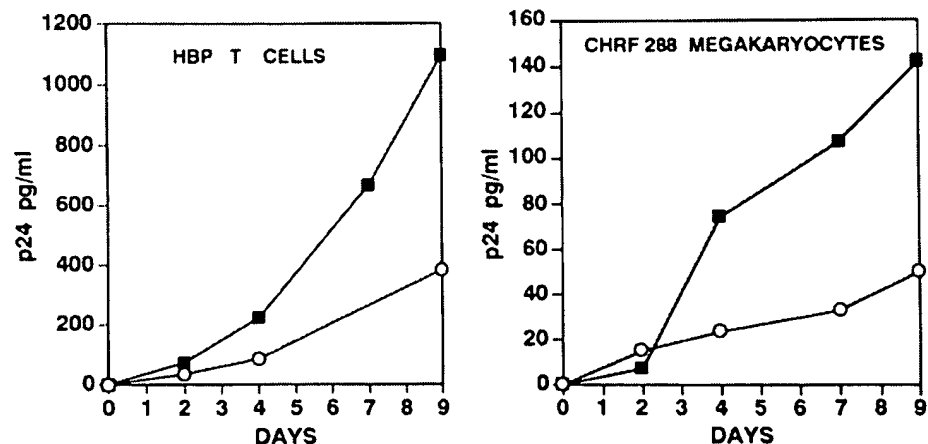
antigen, indicating that the CD4 antigen found on the surface of MGKs is synthesized by these cells and not acquired by adsorption from other cells or that the antibody used to detect the CD4 antigen cross-reacts with a different epitope. We also show that the CD4 on human MGKs is functional as an HIV-1gp120 receptor. Approximately 25% of cells have receptor density similar to that of CD4<sup>+</sup> T cells. Human MGKs are capable of binding wild-type HIV-1 and 55% to 75% of that interaction can be abrogated by pretreatment with either Leu3a, an MoAb that blocks the epitope on CD4 that serves as the receptor for HIV-1gp120, or recombinant soluble CD4 itself. A human MGK line, CHRF-288, can be actively infected with HIV-1<sub>IIIb</sub> as determined by PCR of proviral HIV-1 DNA and p24 antigen production. Infection could be inhibited 65% to 70% with Leu3a. The failure of either the antibody or the rCD4 to completely block binding to MGKs, whereas binding to lymphocytes and monocytes is essentially completely inhibited, suggests that mechanisms other than the interaction between surface CD4 and gp120 can mediate HIV-1 binding and infection in these cells.

Sakaguchi et al<sup>9</sup> have shown that the CMK MGK cell line can be productively infected with HIV-2 ROD, a laboratory strain of HIV-1, and that this can be prevented with Leu3a or rCD4. However, CMK was poorly infected with HIV-1<sub>IIIb</sub>, a second laboratory strain.<sup>9</sup> The MGK line CHRF-288 can be actively infected with HIV-1<sub>IIIb</sub> and prevented with Leu3a. This MGK cell line is unique with respect to other MGK lines (CMK, HEL, K562, LAMA-84, and DAM1) in that it does not contain glycophorin, a red blood cell marker.<sup>9,18</sup> CHRF-288 cells are also reported to be "remarkably homogeneous" in both karyotype and marker expression: 95% of the cells express GPIIb-GPIIIa and PF4.<sup>18</sup>

CD4 was first identified as a differentiation antigen of T-helper cells, in which it is believed to be one of the accessory molecules that make up the T-cell receptor complex. On these cells it is thought to serve the dual function of promoting the adhesive interaction of T cells with antigen-presenting cells and transducing an independent intracellular signal. Signal transduction requires association with a phosphokinase.

The CD4 antigen is present on hematopoietic cells outside the T-cell lineage: circulating monocytes,<sup>20-22</sup> eosinophils,<sup>22</sup> MGKs,<sup>11</sup> and some myeloid tumors.<sup>23</sup> We have been unable

Fig 7. HIV-1p24 secretion by HIV-1-infected HBP-9 (CD4<sup>+</sup> T) cells and CHRF-288 MGKs. Cells ( $1 \times 10^6$ ) were incubated in RPMI in the absence or presence of Leu3a. Cells were then pelleted, washed, and incubated with cell-free HIV-1<sub>IIIb</sub>. Aliquots of the supernatant were assayed for HIV-1p24 for 9 days at 37°C.



to detect CD4-associated phosphokinase activity in CD4<sup>+</sup> monocytes or myeloid tumors. Therefore, it is possible that, in some cells, CD4 may serve only as an adherence receptor. CD4 expression can be modulated in various tissues. In animals in which class II major histocompatibility complex (MHC) expression has been eliminated, CD4 expression in the thymus is dramatically reduced and CD4<sup>+</sup>/CD8<sup>+</sup> (double-positive) cells never develop.<sup>24</sup> CD4 expression on human eosinophils is inducible by granulocyte-macrophage colony-stimulating factor,<sup>25</sup> which had earlier been shown to be a potent regulator of eosinophil function.<sup>26</sup> CD4 expression is downregulated by phorbol esters<sup>27</sup> or by exposure to 1,25-dihydroxyvitamin D<sub>3</sub>.<sup>28</sup> Human monocytes appear to have an intracellular pool of CD4 that is available for expression on their surface.<sup>22</sup>

Expression of CD4 mRNA or antigen by MGKs seems to be unrelated to their size or ploidy content, which would suggest that this molecule does not have a developmental role in the maturation and differentiation of this cell lineage. It is possible that MGK CD4 expression is modulated by other stimuli, as is the case with the above-mentioned tissues.<sup>22,24-28</sup> Alternatively, heterogeneous expression could represent a subpopulation of MGKs. Serologic heterogeneity with respect to expression of ABH-antigens has been shown previously.<sup>29</sup> Although the ABH antigens are not direct gene products, the distribution of high and low expressors seemed to be clonal in MGK colonies. No functional differences between high and low expressors have been described.

The role of CD4 on MGKs is unknown. We have suggested that it may act as an adhesive protein or it may mediate other intercellular interactions,<sup>11</sup> but direct evidence for this is lacking. Whatever its function on normal human MGKs may be, it is clear that CD4 is present on the surface of some MGKs and is capable of providing a receptor for HIV-1 binding.

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## Effects of virion surface gp120 density on infection by HIV-1 and viral production by infected cells

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### Abstract

The quantity of envelope glycoprotein molecules (Env) on HIV-1 particles is still an issue of debate and, depending on the strain of virus and the nature of the producer cells, it can vary greatly. Here, we have attempted to address how Env density influences HIV-1 fitness. To this aim, we have produced HIV-1-derived viral particles with various amounts of R5 Env (low Env: Env<sup>lo</sup>; high Env: Env<sup>hi</sup>), using a regulatable expression system. The infectivity was assayed on human cells, engineered to express the HIV receptor CD4 and the co-receptor CCR5, as well as on peripheral blood lymphocytes and macrophages. In these experiments, low levels of Env were sufficient for cell infection, albeit at low efficiency. Increasing the amount of Env resulted in cooperatively improved infectivity, but a threshold was rapidly attained, indicating that only a fraction of Env was required for efficient infection. Unexpectedly, Env incorporation beyond what gives maximal infection transiently stimulated the expression of proviral genes, as well as retrovirus production, in newly infected cells. This was likely a consequence of induced NF- $\kappa$ B activity, as this transcription factor is triggered by Env<sup>hi</sup>, but not by Env<sup>lo</sup>, virions. Thus, our data suggest that one major effect of high Env density on the surface of HIV may not be better infection yields but rather improved viral production by newly infected cells.

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**Keywords:** HIV-1; Env; Envelope glycoprotein; Infection; LTR activation; NF- $\kappa$ B

### Introduction

HIV-1 is the primary cause of AIDS and associated diseases (Piot et al., 2001; Pomerantz and Horn, 2003; Stevenson, 2003; Weiss, 2001). Its envelope glycoprotein (Env) is responsible for virus binding to target cells, as well as for mediating fusion between the viral envelope and the host cell membrane (Poignard et al., 2001). HIV Env is a heterodimer of two non-covalently linked glycosylated

subunits (gp41 and gp120) proteolytically produced from the same precursor (gp160) (Poignard et al., 2001). gp120 (or SU) is a surface molecule responsible for specific virus attachment to cells, whereas gp41 is a transmembrane protein that tethers Env to the viral envelope and carries the fusion activity. gp120 and gp41 can dissociate, causing shedding of gp120 from the viral surface. Although this mechanism is probably less efficient than initially believed (Chertova et al., 2002), it has been proposed to reduce virion infectivity (McKeating et al., 1991; Poignard et al., 2001). Envs trimerize to form the viral spikes, visible in electron microscopy, which are thought to be the functional units for specific binding of viruses to cells (Poignard et al., 2001).

The interaction of Env with its protein partners is relatively well documented. Within immature virions, the

**Abbreviations:** PBS, Phosphate-buffered saline; Dox, doxycycline; Env, envelope glycoprotein; MWCO, molecular weight cut-off.

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cytoplasmic tail of gp41 interacts with Gag, which reduces Env fusion activity until capsid maturation (Wyma et al., 2004). With regard to the infection process, gp120 binds to the CD4 molecule, which is the primary HIV receptor found on a variety of cells, including T lymphocytes, dendritic cells and macrophages/monocytes (Berger et al., 1999). Different chemokine receptors serve as co-receptors, the main ones being CCR5 and CXCR4, which permit infection by the R5 and X4 HIV-1 isolates, respectively (Baribaud and Doms, 2001; Berger et al., 1999). The change in HIV-1 cellular tropism plays an important role in disease progression and is driven, at least in part, by the selective pressure of the immune system acting in conjunction with the natural variation of the HIV genome (Cohen et al., 1997; Moore et al., 1997). The primary clinically latent stages are associated with R5 species, whereas during the disease X4 viruses may emerge (Cohen et al., 1997; Moore et al., 1997; Paxton and Kang, 1998). The association of gp120 with CD4 elicits a conformational change in Env, unveiling the co-receptor binding site. Then, the association of the gp120–CD4 complex with the relevant co-receptor results in further structural rearrangements in gp41, which in turn permit fusion between the viral and the host cell membranes (Berger et al., 1999; Pognard et al., 2001).

Importantly, intracellular signaling cascades are triggered by the interaction of the virus with both CD4 and the co-receptors (Freedman et al., 2003; Popik and Pitha, 2000; Stantchev and Broder, 2001). However, how this signaling affects the physiology of infected cells and the establishment of viral infection is not yet completely clear. Conversely, the infected cell can influence the expression of proviral genes. Together with viral components, cellular transcription factors interact with the *cis*-acting elements of the HIV promoter and regulate gene transcription according to physiological changes, such as cell stimulation or induction of division and differentiation (Pereira et al., 2000; Roebuck and Saifuddin, 1999; Rohr et al., 2003). Of particular note, two NF- $\kappa$ B transcription factor binding sites reside within the HIV-1 LTR. Although not absolutely necessary for basal transcription, they respond to cellular activation signals by stimulating LTR activity and, thereby, increase the rate of viral production (Berger et al., 1999).

The initial steps of infection are qualitatively well understood, whereas the quantitative parameters are still relatively ill defined. The number of CCR5 molecules required for a successful interaction with Env has been shown to depend on the availability of CD4 (Kuhmann et al., 2000) and determines post-entry efficiency of R5 HIV-1 infection (Lin et al., 2002). However, the number of Env trimers actually required to mediate infection is still unclear and the quantity of Env on the surface of HIV-1 is, in itself, a topic of controversy. Electron microscopic (EM) analysis initially suggested that approximately 72 spikes were incorporated into virions (Gelderblom, 1991). On the basis of a biochemical analysis, a lower number (7–14) of spikes per virion was also proposed (Chertova et al., 2002; Zhu et

al., 2003). Other data consistent with this finding were also published but, in addition, described significantly lower Env contents for several laboratory-adapted strains and primary isolates (Hammonds et al., 2003). In these experiments, the Env value was deduced from the measurement of the Gag-to-Env ratio, assuming that HIV particles contain 1200–2500 Gag copies. Recent cryo-EM and scanning transmission EM, however, rather indicated a content of 5000 Gag proteins per virions (Briggs et al., 2004), suggesting that the amount of Env may have been underestimated by 2–4 fold in the Chertova et al.'s, Zhu et al.'s, and Hammonds et al.'s reports. Whatever the case, the number of Envs that can be incorporated into viral particles is limited, including for those produced by 293T cells, which are classically used to generate pseudovirions, with a minimal Gag-to-Env ratio of 45:1 to 70:1 (Hammonds et al., 2003). Interestingly, the limiting factor is not gp160 expression level by HIV-producing cells but another still-to-be-identified mechanism (Hammonds et al., 2003).

Here, we have aimed at determining to which extent Env density at the virion surface is a critical parameter of HIV infectivity. As various viral components can influence virus infection/activity in a difficultly quantifiable manner, we set out to investigate the effects of differences in SU quantity while keeping all other viral factors constant. HIV-1 particles with different Env contents were produced in 293 TetOn cells under standardized conditions and both their efficacy of infection and proviral expression in newly infected cells were subsequently compared. Our data support the idea that a limited amount of Env molecules is sufficient to achieve infection, albeit at low efficiency, and a cooperation between a small fraction of Envs is sufficient to obtain maximal efficiency. Importantly, increasing the quantity of Env above the level that yields the maximum infectivity induces expression of LTR-driven genes in newly infected cells, most probably via protracted NF- $\kappa$ B pathway activation. The potential consequences of these observation on the initial events of cellular infection by HIV and on viral production by newly infected cells are discussed.

## Results

### *Infection efficiency of virions with different R5 Env amounts*

As R5 viruses are the predominant species during viremia establishment in infected individuals, we chose to study the AD8 strain R5 Env (Cho et al., 1996). Since it is not possible to purify homogenous populations of natural HIV particles displaying different amounts of Env, we resorted to a replication-incompetent EGFP reporter gene-expressing HIV-1 vector (pHR-TE), which achieves one round of infection and permits direct scoring of infected cells through fluorescence analysis. Virions were produced in 293 TetOn cells, stably expressing the doxycyclin (Dox)-activatable rtTA transcription transactivator (Baron and

Bujard, 2000), after cotransfection with (i) CMV $\Delta$ R8.9, a HIV-1-derived packaging plasmid (Zufferey et al., 1997), (ii) the HIV-1 pHR-TE vector expressing constitutively the CMV promoter-driven EGFP gene, and (iii) PM636, carrying a rtTA-responsive AD8 Env gene (Fig. 1A) in the presence of increasing concentrations of Dox. To verify Env dose-dependent expression, immunoblotting experiments were performed 48 h after transfection, using a specific anti-gp120 Env antiserum. Env quantities in cell lysates, as well as in extracts from CaCl<sub>2</sub>-precipitated secreted proteins, varied as a function of Dox. However, due to the intrinsic leakiness of the tetracycline expression system, a basal level of Env expression could be observed, even in the absence of Dox, (Fig. 1B). Variations in absolute quantities of Env at a given Dox concentration were observed between the different experiments (for example, compare Fig. 1B with Fig. 2C), but they did not influence the final conclusions. Flow cytometry analysis of trans-

fected cells was also performed to analyze cell surface-associated AD8 gp120 at the individual cell level (Fig. 1C). Transfected cells were found in sharp peaks of fluorescence, whereas non-transfected cells showed no fluorescence. The mean fluorescence ratio of these peaks (4.3 between 1000 and 100 ng Dox/ml and 3 between 100 and 10 ng Dox/ml) was comparable to the ratio of AD8 gp120 Env abundances (4 between 1000 and 100 ng Dox/ml and 3.4 between 100 and 10 ng Dox/ml) assayed by cell extract immunoblotting analysis (Fig. 1Ba), demonstrating a Dox dose-dependent expression of AD8 gp120 by transfected cells.

Hos-CD4-CCR5<sup>hi</sup> cells are human osteogenic sarcoma cells engineered to express CD4 and high quantities of CCR5 for easy R5 virus infection studies and sorted for homogenous CCR5 expression (Kato et al., 1999; Lin et al., 2002). Standard virus assays (cfu assay) in which serial dilutions of viral suspensions were placed in the presence of an excess of target cells were conducted (Fig. 2A) and viral

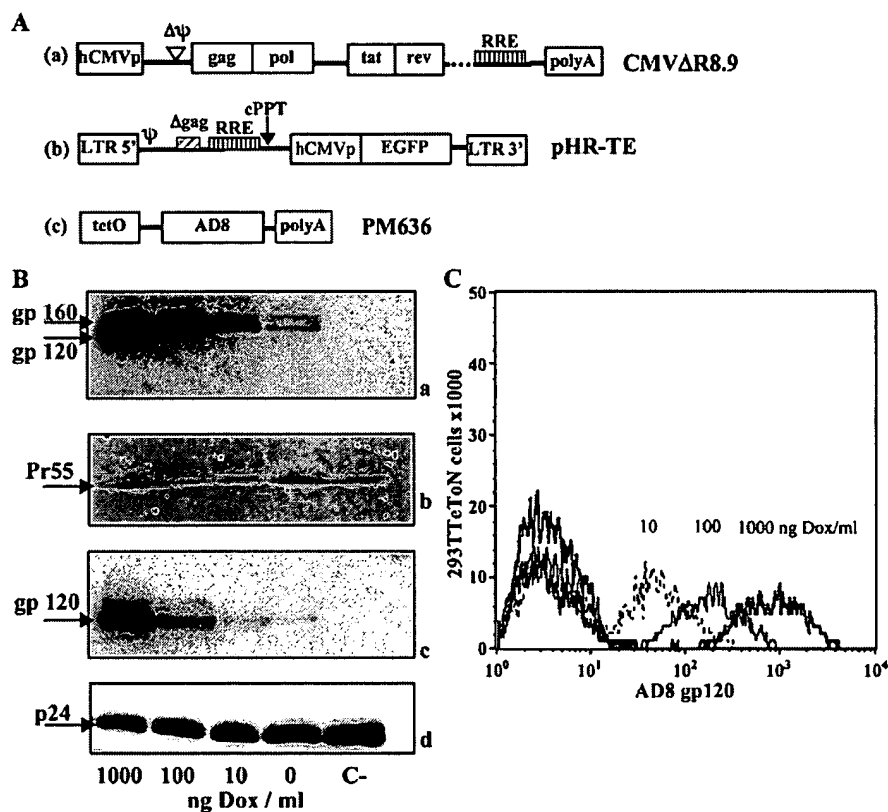


Fig. 1. HR-TE virions with different Env amounts. (A). Structure of expression plasmids. CMV $\Delta$ R8.9 (a) is an HIV-1-derived packaging plasmid with no *env* gene and no packaging signal. HIV-1 LTRs have been replaced by both the constitutive CMV promoter and a heterologous polyadenylation signal. pHR-TE (b) is an HIV-1-based vector expressing the EGFP gene under the control of the CMV promoter. PM636 (c) carries a rtTA-responsive AD8 Env gene. (B). Env abundance in virus-producing cells and viral particles as a function of Dox concentration. 293 TetOn cells were transiently cotransfected, in the presence of various concentrations of Dox, with CMV $\Delta$ R8.9, pHR-TE and PM636. Env abundance in cell extracts and in purified virions was assayed by immunoblotting using an anti-gp120 antiserum 2 days post-transfection. Both the gp160 Env precursor and the processed gp120 are detected in cells, (a) whereas only gp120 is detected in viral particles (c). The identity of gp160 and gp120 in cells was confirmed by electrophoresing cellular and viral extracts in parallel through the same gel before immunoblotting analysis (not shown). The blots were stripped and reprobed with an anti-Gag protein antibody detecting the HIV-1 Pr55Gag precursor in cells (b) and the processed p24Gag capsid protein in virions (d) to verify that comparable amounts of viral proteins were analyzed in all tracks. C- indicates control cells transfected with pHR-TE and CMV $\Delta$ R8.9. Densitometer scanning analysis was performed using appropriately exposed luminograms. (C) Flow cytometry analysis of Env-expressing 293 TetOn cells. Cells,  $5 \times 10^5$ , corresponding to the immunoblotting experiment presented in B (a) were analyzed by flow cytometry using the 2G12 anti-gp120 monoclonal antibody.

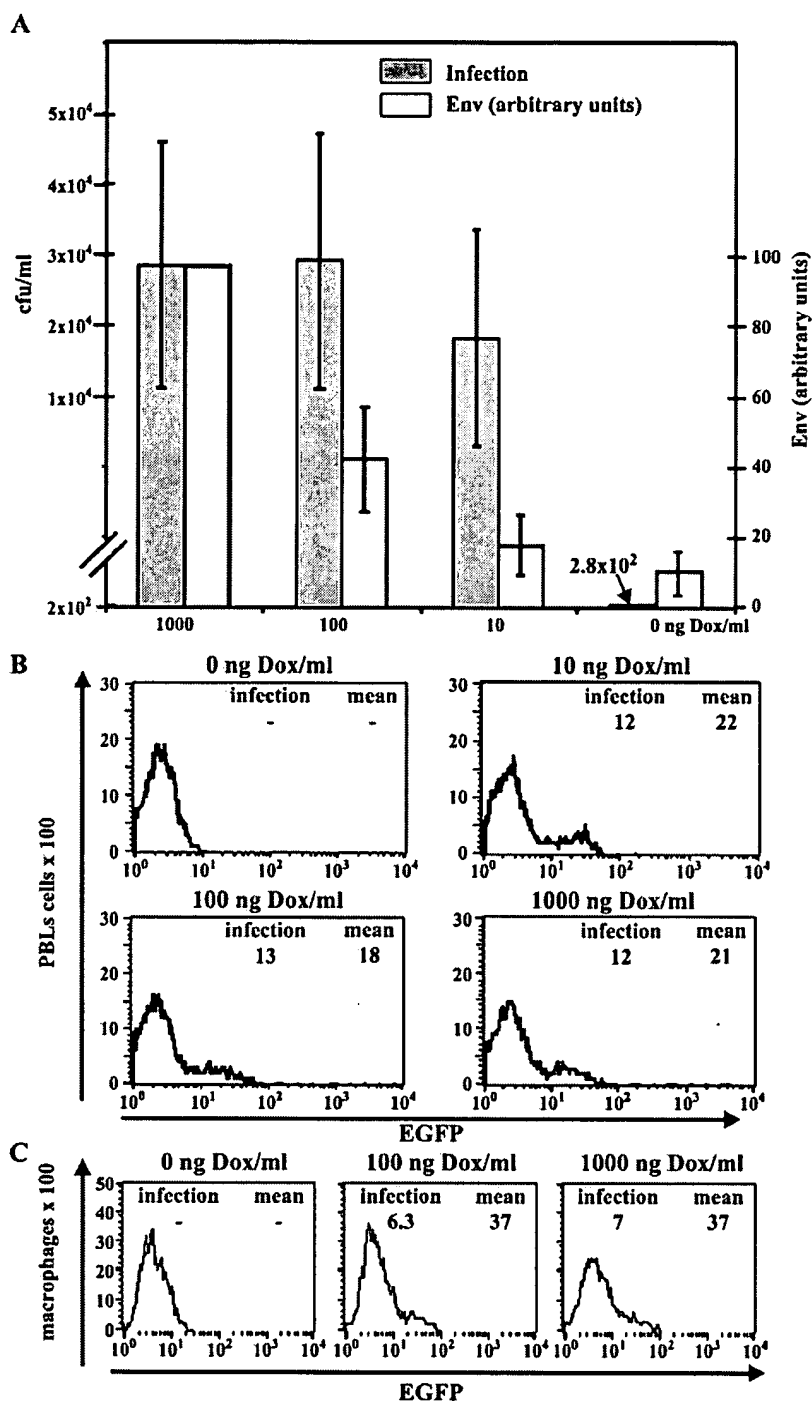


Fig. 2. Infection of Hos-CD4-CCR5<sup>hi</sup> cells, PBLs and macrophages by HR-TE virions. (A). CfU assay on Hos-CD4-CCR5<sup>hi</sup> cells. The virions with different Env contents in Fig. 1 were used for triplicate infections of Hos-CD4-CCR5<sup>hi</sup> in the cfU assay. Relative Env abundances are the average of three immunoblotting assays and were deduced from densitometer scanning analysis of appropriately exposed luminograms. One hundred percent corresponds to Env content of viruses prepared in the presence of 1000 ng Dox/ml, which was the maximal abundance that could be obtained. Error bars represent the standard deviation. (B). Infection of PBLs. The same viruses as those used in A were used to infect human PBLs. Infection was monitored through EGFP fluorescence assay by flow cytometry. Two parallel experiments using  $2 \times 10^6$  cells were conducted with the same final outcomes. Both the percentages of infected cells and the mean fluorescence values per cell are indicated in the experiment presented. (C) Infection of macrophages. Three new HR-TE virion batches were produced in the presence of 0, 100, and 1000 ng Dox/ml. Their relative Env contents were 1, 2, and 16. The two virions preparations with the highest Env contents were equally infectious in the cfU assay (approximately  $10^4$  cfu/ml), whereas that with the lowest Env content was 100-fold less infectious. Infection of differentiated macrophages obtained was monitored by flow cytometry as in B using  $3 \times 10^5$  cells.

titers were plotted against relative Env abundances. No infection was detected in the presence of particles produced in the absence of the PM636 Env expression vector. Moreover, there was no linear relationship between Env abundance and infection efficiency. The 2-fold increase in Env levels between 0 and 10 ng Dox/ml conditions resulted in a 100-fold improvement of infectivity. However, the 2.6-fold (between 10 and 100 ng Dox/ml) and the 2.5-fold (between 100 and 1000 ng Dox/ml) rise in Env densities did not result in any significant increase in infection. Similarly, when infections were analyzed by flow cytometry (not shown), the same percentages of cells (approximately 30%)

were positive whether viruses were produced in the presence of 10, 100, or 1000 ng Dox/ml, whereas no infection was detectable with viruses produced in the absence of Dox. As flow cytometry is a less sensitive method than the cfu assay, this lack of infection is consistent with the 100-fold titer difference seen in the cfu experiment.

We next conducted experiments with natural HIV-1 target cells, that is, human peripheral blood lymphocytes (PBL) and primary macrophages. Suboptimal transduction conditions were chosen to avoid potential saturation, while still obtaining a reference level well above non-specific background. Using the same viral preparations as in Fig. 2A,

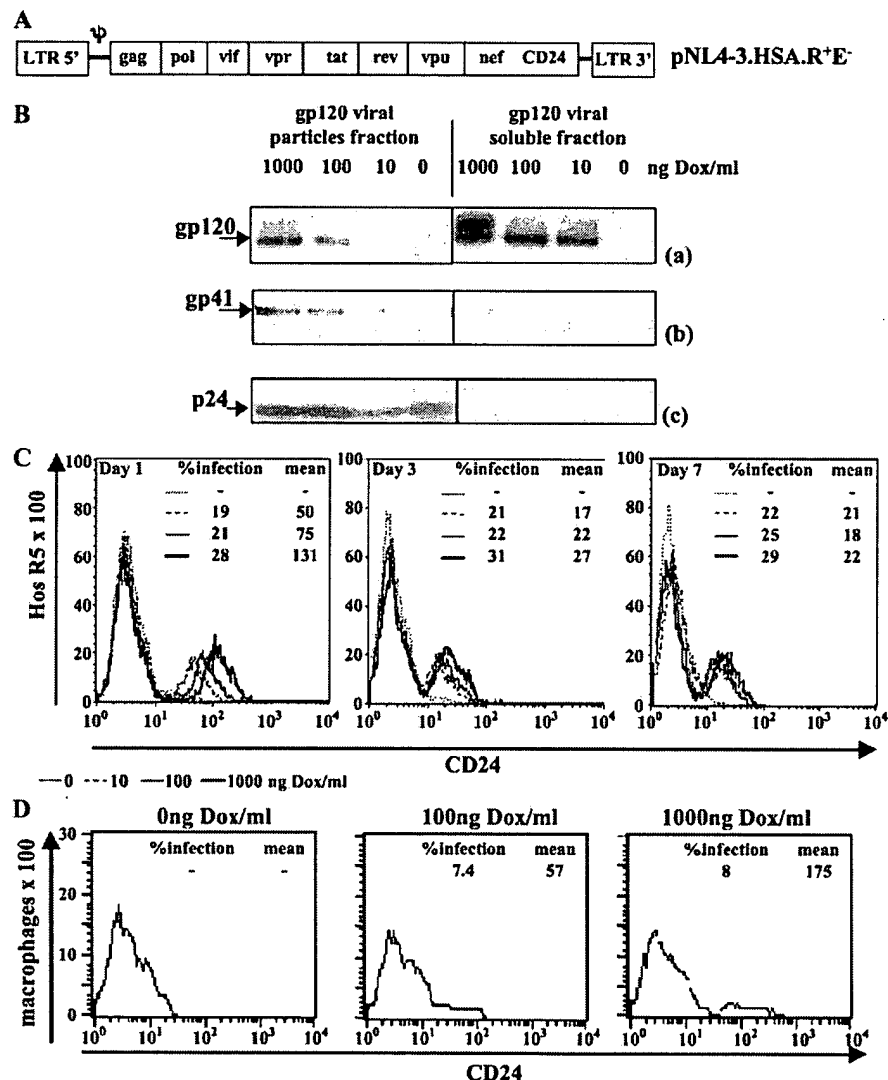


Fig. 3. Infection of Hos-CD4-CCR5<sup>hi</sup> and macrophages by NL4-3.HSA.R+E- virions. (A) pNL4-3.HSA.R+E- vector. The vector contains the entire HIV-1 genome where (i) the *env* gene is mutated and inactive and (ii) CD24 is inserted within the *nef* gene. (B) Preparation of soluble gp120-free virions displaying different Env amounts. Culture supernatants containing NL4-3.HSA.R+E- virions with different Env amounts were filtered through  $1 \times 10^6$  MWCO membranes. Concentrated virions and filtrates were tested by immunoblotting for the presence of gp120, gp41 and p24 proteins using specific antibodies. (C) Infection of Hos-CD4-CCR5<sup>hi</sup> cells. Hos-CD4-CCR5<sup>hi</sup> cells,  $5 \times 10^4$ , infected with the virions presented in B and analyzed by flow cytometry for CD24 expression at different times post-infection. Relative amounts of Env in virions prepared in the presence of 0 (—), 10 (---), 100 (—) and 1000 (—) ng Dox/ml were 1, 2, 5 and 12, respectively. The experiment presented is representative of 3 independent experiments. (D) Infection of macrophages. Human macrophages were infected using virus preparations produced in the presence of 0, 100, and 1000 ng Dox/ml and displaying relative Env ratios of 1, 2, and 18, respectively. Flow cytometry analysis was performed as in C using  $3 \times 10^5$  cells.

comparable fractions of PBL (12–13%) were infected by virions produced in the presence of either 10, 100, or 1000 ng Dox/ml, whereas virions produced in the absence of Dox did not detectably infect the cells. (Fig. 2B). New batches of virus were prepared, and likewise, we did not observe any infection of macrophages by virions produced in absence of Dox, although viruses with 2- and 16-fold higher Env contents appeared equally infectious (Fig. 2C).

Taken together, our data indicate that (i) a limited Env amount is sufficient to permit infection by HIV-1, albeit at low efficiency, (ii) a slight increase in Env incorporation results in a dramatic improvement of cell infection indicative of cooperation between Envs, and (iii) a threshold of Env density above which no improvement in cell infection is observed is rapidly reached.

#### *Differential HIV-1 LTR activation in cells infected by virions with different Env amounts*

Binding of soluble and cell membrane-exposed gp120 to HIV receptor and co-receptors can activate intracellular signaling (Freedman et al., 2003; Popik and Pitha, 2000; Stantchev and Broder, 2001). We, therefore, assessed here whether virions with different Env levels could alter signaling as well and, thereby, influence HIV provirus expression in recently infected cells. The pNL4-3.HSA.R+E-HIV-1-derived vector (Fig. 3A) contains the entire HIV genome with a mutated, non-functional *env* gene and the CD24 (HSA) cell surface glycoprotein cDNA, inserted into the *nef* gene and consequently transcribed from the LTR. Importantly, the change in the promoter controlling reporter protein gene transcription allows for both the identification of infected cells and the assay of provirus expression levels in the same CD24-based flow cytometry experiment. To concentrate the virus and exclude small molecules, including soluble Env and Dox, culture supernatants from transfected 293 TetOn cells were filtered through  $1 \times 10^6$  MWCO membranes (Fig. 3B). Hos-CD4-CCR5<sup>hi</sup> cells were infected and flow cytometry analysis was performed 1, 3, and 7 days later (Fig. 3C). As above (Fig. 2), retroviruses produced in the absence of Dox gave no detectable infection, albeit low-efficiency transduction occurred in the cfu assay (not shown). Again, the percentages of infected cell were in the same range for all other conditions of production, regardless of the day of analysis. However, 1 day post-infection, CD24 expression levels were elevated when Env incorporation was high: in the presence of 100 and 1000 ng Dox/ml, CD24 was 50% and 130% higher, respectively, than with 10 ng Dox/ml. This effect is no longer detectable after 3 and 7 days. Pseudotransduction of cell surface molecules from retrovirus-producing cells to the membrane of infected cells has already been reported to bias gene transfer experiments (Gallardo et al., 1997). However, very low and identical basal levels of CD24, the level of which was not reduced in the presence of AZT, were associated with purified virions, regardless of the Dox concentration (not shown), indicating

the absence of pseudotransduction by the lentiviral vectors used. Hence, co-transport of CD24 with Env is not accountable for the effects observed on day 1. Southern blotting analysis of high molecular weight DNA prepared from infected cell showed that comparable amounts of proviruses were generated upon infection with virions produced in the presence of 10, 100, and 1000 ng Dox/ml (data not shown). Also, this was further demonstrated with real-time PCR, confirming that the viral copy numbers in the differently infected cells were in the same range (Table 1). Hence, this difference in CD24 expression is not a result of more productive infections. Therefore, we tested the hypothesis that Env<sup>hi</sup> virions could stimulate HIV gene expression more efficiently than Env<sup>Lo</sup> virions.

For this purpose, we looked at the LTR-activation in natural target cells for HIV-1, infected with Env<sup>Lo</sup> and Env<sup>hi</sup> viruses. To obtain differentiated macrophages, purified monocytes were induced by VitD3, a differentiating agent for myelomonocytic cell that does not trigger HIV-1 expression (Goletti et al., 1995). The cells were incubated with virions, which carried relative Env contents of 1 (0 ng Dox/ml), 2 (100 ng Dox/ml) and 18 (1000 ng Dox/ml), respectively. Consistent with our previous results, we did not detect any infected cells using Env<sup>Lo</sup> particles, whereas infection efficiencies of the other two virus (100 and 1000 ng Dox/ml) preparations were comparable (Fig. 3D). Again, there was a significant (3-fold) difference in CD24 expression between the two infectious samples. Thus, stimulation of proviral expression by high Env density virions is not restricted to Hos-CD4-CCR5<sup>hi</sup> cells but also pertains to natural HIV targets.

#### *Higher viral protein production by cells newly infected by virions with high Env content*

Next, we asked if higher proviral expression in recently infected cells by Env<sup>hi</sup> virions resulted in increased viral production. Three NL4-3.HSA.R+E- viral batches with relative Env contents of 1, 2, and 12 were produced. Again, the virus preparation with lowest Env density was poorly infectious, whereas the other two were equally infectious on Hos-CD4-CCR5<sup>hi</sup> cells. As expression of the viral proteins in infected cells are driven by the LTR, the amount of Gag protein precursor (Pr55Gag) in cell extracts directly reflects provirus expression, whereas that of mature Gag (p24Gag) in culture supernatants mirrors viral particle release from

Table 1  
Quantitative PCR on genomic DNA from cells infected with viral particles expressing different quantities of Env

Sample (n = 6)	0 ng/ml Dox	10 ng/ml Dox	100 ng/ml Dox	1000 ng/ml Dox
Provirus AU <sup>a</sup>	6	769 ± 188	943 ± 82	1004 ± 89

<sup>a</sup> Arbitrary units: provirus-to-control DNA ratio (see Materials and methods). The amplified pro-virus signals are expressed as a function of CCR5.

cells. p24Gag and Pr55Gag were assayed by immunoblotting 24 h post-infection and only detected when using the two most infectious viral preparations. As predicted, the most elevated protein levels were found in cells infected with virus bearing maximum Env density (Fig. 4A).

Similar experiments were conducted with human primary macrophages, using viruses with relative Env levels of 1, 2, and 18. Pr55Gag was detectable, albeit at a low level, exclusively in cells infected by virions expressing the highest levels of Env, whereas p24Gag was not detected in any conditions (Fig. 4B). This is not surprising, as production of viral particles by infected macrophages occurs

at a much slower rate than that of infected Hos-CD4-CCR5<sup>hi</sup> cells (Li et al., 1999; Naif et al., 1999). Analysis to detect viral particle release at later time points could not be performed because of culture degeneration. Nevertheless, these data indicate that viral expression was increased in cells infected by virions with a high Env content.

#### *Different activation of the NF- $\kappa$ B pathway by Env<sup>lo</sup> and Env<sup>hi</sup> HIV-1 virions*

As the NF- $\kappa$ B pathway is implicated in HIV-1 LTR activation, we finally investigated whether this cascade could be differently affected in cells infected by either Env<sup>hi</sup> or Env<sup>lo</sup> virions. Hos-CD4-CCR5<sup>hi</sup> cells were transfected with reporter plasmids expressing the firefly luciferase gene from either a minimal constitutive (conaluc) or a NF- $\kappa$ B-inducible ((Ig $\kappa$ )<sub>3</sub>-conaluc) conalbumin promoter. The cells were incubated with two populations of NL4-3.HSA.R+E- particles, from where soluble gp120 had been excluded. The virus preparations displayed a 6-fold difference in Env content, but had the same infection efficiency (not shown). Luciferase activity was assayed in cell extracts prepared 6, 24 and 48 h after a 6-h infection. No (1.2-fold) stimulation of the NF- $\kappa$ B pathway was observed when infections were performed with the lowest Env density particles (Fig. 4C). In contrast, NF- $\kappa$ B activity was stimulated at all time points tested with a peak of activity (8-fold stimulation) 24 h post-infection using the highest Env density virus preparation. This activity was comparable to that measured 6 h post-stimulation by TNF- $\alpha$  in control experiments (not shown). We also assayed pseudotyped NL4-3.HSA.R+E- virions expressing 4-fold different amounts of the amphotropic murine leukemia virus Env. Both vectors were equally infectious on Hos-CD4-CCR5<sup>hi</sup> cells, indicating that this particular Env can also be incorporated in apparent excess with regard to maximal infection efficiency. However, neither induced NF- $\kappa$ B activity (not shown), pointing to a specific effect of HIV Env with regard to differential activation of this pathway.

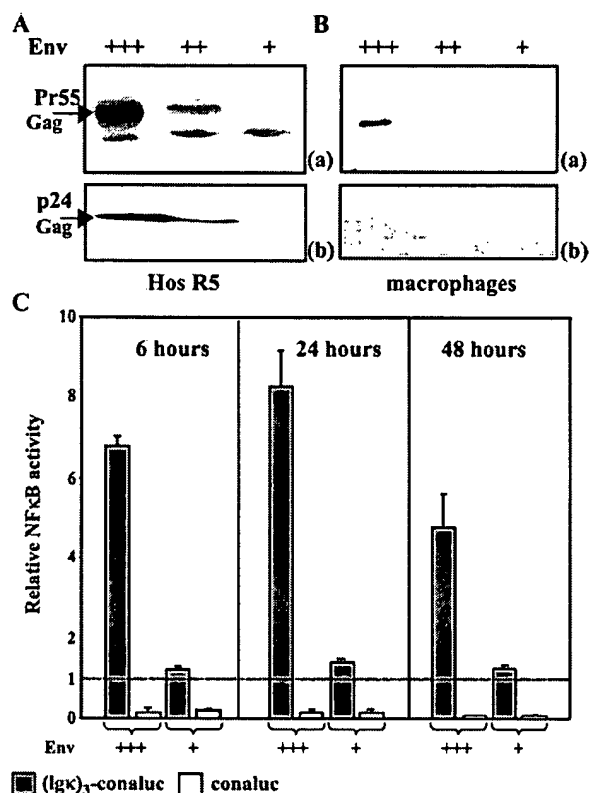


Fig. 4. NF- $\kappa$ B activation, provirus expression and viral particles production in cells infected by virions with different Env contents. (A) Viral particles production by newly infected Hos-CD4-CCR5<sup>hi</sup> cells. Hos-CD4-CCR5<sup>hi</sup> cells were infected with virions displaying 1 (+), 2 (++) and 12 (+++) relative levels of Env. The latter two preparations were equally infectious and the former one poorly. Pr55Gag and p24Gag abundances were assayed by immunoblotting using a specific anti-Gag antiserum in cell extracts and purified viral particles, respectively, 24 h post-infection. (B) Proviral expression in newly infected macrophages. The experiments were conducted as in B except that relative amounts of Env in virion preparations were 1 (+), 2 (++) and 18 (+++). (C) NF- $\kappa$ B activation in Hos-CD4-CCR5<sup>hi</sup> cells. Hos-CD4-CCR5<sup>hi</sup> cells were transiently transfected with conaluc and (Ig $\kappa$ )<sub>3</sub>-conaluc plasmids. Twenty-four hours later, they were infected for 6 h using two populations of NL4-3.HSA.R+E- virions showing a 6-fold difference in Env content but similar infectivity. Luciferase activity was measured 6, 24, and 48 h post-infection. The presented data are the average of three experiments. (+) and (+++) correspond to virions expressing low and high Env amounts, respectively. The reference activity of 1 corresponds to the basal NF- $\kappa$ B activity in non-infected Hos-CD4-CCR5<sup>hi</sup> cells assayed using (Ig $\kappa$ )<sub>3</sub>-conaluc. The error bar represents the standard deviation.

## Discussion

Here, we have evaluated how different Env contents can influence virus fitness and viral production by newly infected cells as this might help predicting the propagation of certain virus isolates and, ultimately, the progression of AIDS.

#### *Influence of Env density on cell infection*

Several lines of evidence have long supported the idea that a large fraction, if not most, of Env on virions is required for cell infection by HIV. For example, initial infection-blocking experiments using soluble CD4 (sCD4) (Layne et al., 1990) and neutralizing monoclonal antibodies

(mAb) (Parren and Burton, 2001; Schonning et al., 1999) have suggested that approximately 50% and 60%, respectively, of natural Env levels were required for infection. Moreover, major losses of infectivity as virions ages were observed in association with only relatively small losses of gp120 from virions (McKeating et al., 1991).

Contrasting with this view, our data rather suggest that (i) a small fraction of the Env amount that can be incorporated by HIV-1 particles is sufficient for infection, albeit at low efficiency, (ii) a higher, but still limited, number of Envs can cooperate for maximal infection and (iii) an “apparent” vast excess of Env is incorporated into viral particles. Although it is difficult to formally rule out that low-efficiency infection by Env<sup>lo</sup> viruses reflect infection by a small fraction of viruses with a higher Env content, it is important to underline that similar observations were previously made with murine leukemia viruses (Bachrach et al., 2000). Moreover, using mAbs as molecular probes, Pognard et al. (2003) recently demonstrated that a fraction of Envs expressed on primary HIV-1 isolates is non-functional, which also strengthens the notion that only part of envelope glycoproteins exposed on the surface of HIV particles is sufficient for infection. Additional work is still needed to precisely quantify this fraction and to determine whether it corresponds to misfolded or non-multimerized molecules. However, this will constitute a difficult task for at least two reasons. First, the number of Env carried by natural HIV particles is still a matter of controversy (see Introduction). Second, if quantification of the number of Env molecules on high Env density-displaying virions is technically feasible, that on Env<sup>lo</sup> particles still necessitates methodological improvements. Importantly, we do not feel that our Env<sup>hi</sup> viruses corresponded to viruses with supraphysiological contents of Env for two reasons: (i) a limit to the Env amount that can be incorporated into HIV-1 virions is rapidly attained and corresponds to levels found in several natural strains and isolates, albeit the molecular reasons for this still remains to be determined (Hammonds et al., 2003) and (ii) a preliminary analysis has shown that the maximal amount of Env incorporated in our Env<sup>hi</sup> virions was comparable to that harbored by a primary isolate (not shown). Although comparison with a larger sampling of primary isolates and laboratory strains is still required for precise quantification, these arguments led us to speculate that only a few spikes (and maybe just one) are sufficient for low efficiency infection.

The mechanisms underlying synergy between a few Envs for efficient infection are still unknown and may be multiple. Further work will address whether they involve concerted actions between Env monomers within spikes and/or cooperation between spikes for recruiting several receptors/co-receptors or forming more efficient fusion pores. Cooperation between SU and TM Env components and/or between Env monomers is possible as different Env mutants can reciprocally complement their defects within mixed oligomers (Salzwedel and Berger, 2000). Recent

electron microscopy analysis revealed relatively uniform spacing of Env trimers at the surface of HIV virions and provided no evidence for physical association of spikes (Zhu et al., 2003). However, the authors did not formally exclude the possibility of functional collaboration between spikes as it is not clear yet whether spacing between Envs was due to actual structural constraints imposed by the underlying viral matrix proteins or to technical reasons, such as insufficient number of analyzed particles, insufficient sensitivity of the method or unavoidable deformations resulting from electron microscopy sample preparation (Zhu et al., 2003). Finally, it is worth underlining that the dynamics of Env multimerization on the viral surface has, thus far, not been investigated in-depth. Therefore, it is possible that Env oligomers may dissociate and reassociate on the viral surface and that cooperativity reflects facilitation of functional spike formation above a certain Env density threshold.

In our experiments, approximately 80% of Envs incorporated into virions appeared dispensable for cell infection. An important question is whether a similar fraction is also dispensable in natural *in vivo* situations. At that stage of investigation, we do not exclude that it may differ significantly and vary among the different virus strains for a variety of reasons. Firstly, it has been shown that expression of CD4 in virus-producing cells can reduce the infectivity of HIV virions (Arganaraz et al., 2003; Levesque et al., 2003), presumably by blocking the CD4-binding domain of Env via the formation of CD4–Env complexes at the surface of HIV. As no CD4 is expressed by 293T cells, this factor was not taken into consideration in our experiments. It is therefore possible that the presence of CD4 *in vivo* increases the dependence upon incorporation of higher amounts of Env and, thereby, affects the fraction of Env not necessary for/involved in the infection process. Production of HIV particles by 293T cells expressing varying amounts of CD4 will allow to address this issue. Secondly, Env from laboratory-adapted strains usually display higher affinity for CD4 than Env from primary isolates (Kozak et al., 1997; Platt et al., 1997). It is therefore conceivable that a higher number of Env-receptor interactions is required for infection by primary viruses and that, although the densities of CD4 and CCR5 at the surface of the Hos-CD4-CCR5<sup>hi</sup> cells were quite physiological, their relatively high levels of expression have masked this requirement. Experiments with Envs cloned from primary isolates and indicator cells expressing different levels of receptor and co-receptor should help resolving this question.

#### *Possible role(s) for the apparent Env excess*

The reasons for the apparent excess of Env may be multiple and non-exclusive. The most trivial explanation is to keep an Env amount superior to that required for infection to compensate for shedding. Moreover, it must be taken into consideration that, as previously mentioned, a fraction of



Env is likely to be non-functionally exposed at the surface of HIV particles (Poignard et al., 2003). In the specific case of MLVs, we have shown that increasing Env density accelerates the rate at which infectious attachment to cells occurs without affecting the final infection outcome (Bachrach et al., 2000). However, testing whether this also holds true for HIV was impossible because the metastable gp120–gp41 association does not resist the stringency of cell washings used in infection kinetics experiments. Nevertheless, as retroviruses adsorb onto cells in an Env-independent manner (Pizzato et al., 1999) before browsing the cell surface until viral receptor encounter, it is reasonable to assume that high Env density increases the frequency of this and, thereby, accelerates virus entry into cells. This might provide Env<sup>hi</sup> virions with a selective advantage *in vivo* because it reduces the chances of inactivation by simple ageing or by the various clearance systems. Env amounts high above that sufficient for infection may also permit HIV to display highly redundant decoys for neutralizing antibodies. Consistent with this idea, efficient virus neutralization was proposed to require an average of one antibody molecule per spike (Poignard et al., 2001). It has also been proposed that the already mentioned fraction of non-functional Env exposed at the surface of HIV virions may stimulate the generation of non-neutralizing antibody (Poignard et al., 2003). Incorporation of more Env than necessary for infection might, thus, represent an additional advantage for HIV for biasing the humoral antiviral response.

There is ample evidence that HIV-1 virus uses both CD4 and chemokine receptors to induce signaling that both perturb the immune system (via mechanisms largely contributed by soluble gp120) and facilitate the early stages of infection (Freedman et al., 2003; Popik and Pitha, 2000; Stantchev and Broder, 2001). Our work has unveiled another unanticipated selective advantage for Env<sup>hi</sup> HIV virions beyond the establishment of infection, as these, but not Env<sup>lo</sup> particles, transiently stimulated proviral expression in newly infected Hos-CD4-CCR5<sup>hi</sup> cells and primary macrophages. Consistent with the notion that NF- $\kappa$ B is of particular importance for efficient HIV transcription, we observed protracted NF- $\kappa$ B activity in Hos-CD4-CCR5<sup>hi</sup> cells infected with Env<sup>hi</sup>, but not Env<sup>lo</sup>, particles. The Env threshold as well as the signaling mechanisms and kinetics involved deserve further investigations. Whatever the explanation, it is tempting to speculate that infection by Env<sup>hi</sup> viruses may entail better retroviral production *in vivo* (at least for a certain period of time post-infection) and, thereby, higher viral load. Testing whether Env density correlates with virus load and disease progression in infected individuals would be interesting in this respect. Not unexpectedly, we could not observe higher proviral expression upon PBL infection by Env<sup>hi</sup> (not shown) as *in vitro* infection necessitates prior cell activation (Korin and Zack, 1998, 1999), which also induces sustained NF- $\kappa$ B activation. However, it is unlikely that infection of T cells *in vivo*

occurs in the presence of such a high NF- $\kappa$ B activity (Blaak et al., 2000; Eckstein et al., 2001; Zhang et al., 1999). Therefore, it is possible that differential retroviral production may happen upon infection of natural T cells by viruses with different Env levels. Apart from NF- $\kappa$ B, free gp120 is known to alter multiple other signaling cascades via both CD4 and chemokine receptors (Berger et al., 1999). Therefore, we do not exclude that high Env-displaying virions may also stimulate other pathways influencing proviral expression and/or infected cell physiology. Along this line, it is worth noting that, at variance with CD4, which is expressed in a narrow range, CCR5 cell surface abundance varies among individuals and correlates with both R5 HIV-1 infectivity *in vitro* (Platt et al., 1998), virus load (Reynes et al., 2000), and disease progression *in vivo* (Reynes et al., 2001). It would, thus, be interesting to study whether variations in both viral Env- and cell surface CCR5 density can influence HIV propagation via mechanisms, which could non-exclusively include cell entry efficacy and perturbation of signaling pathways.

## Materials and methods

### Plasmids

pRL-TK and pEGFP-C1 are from Clontech. The firefly luciferase (Ig $\kappa$ )<sub>3</sub>-conaluc and conaluc reporter plasmids are described in Munoz et al. (1994) and CMV $\Delta$ R8.9 and pNL4-3.HSA.R+E- in Zufferey et al. (1997) and He et al. (1995), respectively. pHR-TE was constructed by replacing lacZ from pHR-CMVlacZ (Naldini et al., 1996) by EGFP from pEGFP-N1 (Clontech) and PM636 by inserting the AD8 Env gene of pCMVAD8env (Cho et al., 1996) into pUHD-10-3 (Baron and Bujard, 2000).

### Cell lines and primary cells

293 TetOn and Hos-CD4-CCR5<sup>hi</sup> cells are from Clontech and the NIH AIDS Research and Reference Reagent Program (NARRRP), respectively. They were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 units/ml streptomycin, 100 units/ml penicillin, 2 mM L-glutamine and 10% heat-inactivated fetal calf serum (Gibco-BRL) (Hos-CD4-CCR5<sup>hi</sup> cells) or Tet System-Approved Fetal Bovine Serum (Clontech) (293 TetOn cells). Importantly, 293 cells produce little microvesicles that could possibly incorporate viral proteins (Hammonds et al., 2003) and complicate the interpretation of our results. Mononuclear human cells were isolated from informed healthy donor fresh blood samples by Ficoll–Paque density gradient centrifugation. PBLs cells, 10<sup>7</sup>, were induced in 5 ml of RPMI 1640 medium (Gibco/BRL), containing 10% heat-inactivated fetal calf serum, 100 units/ml streptomycin, 100 units/ml penicillin, 2 mM L-glutamine, 5  $\mu$ g/ml of phytohemagglutinin (PHA, Difco) and 10

U/ml of recombinant human IL-2 (Boehringer) for 36 h. PHA and IL-2 were washed away once with RPMI medium before infection. Monocytes were purified from peripheral blood mononuclear cells by negative selection using monoclonal antibodies for CD2, CD7, CD16, CD19, and CD56 and magnetic beads (DynaL Biotech) and seeded at a density of  $3 \times 10^5$  cells/well in 12-well plates with RPMI medium complemented with  $10^{-7}$  M 1- $\alpha$ ,25-Dihydroxyvitamin D3 (VitD3) (Hoffmann-La Roche SA). Purified cells, 97.4%, were CD1a<sup>+</sup>, CD14<sup>+</sup>, CD32<sup>+</sup>, CD64<sup>+</sup>. After 5 days, adherent cells were collected and stained using phycoerythrin (PE)-CD1a and FITC-CD14 (Beckman-Coulter) to confirm that they belonged to the macrophage lineage.

#### *Cell transfection and virus production*

293 TetOn cells were transiently transfected using the calcium phosphate coprecipitation procedure (Sambrook et al., 1989). Cells,  $2 \times 10^5$ , per well of 6-well culture plates (Nunc) were co-transfected using 3  $\mu$ g of each of the relevant plasmids. Sixteen-hour post-transfection, cells were washed three times with phosphate-buffered saline (PBS: 0.15 M NaCl, 0.01 M NaPO<sub>4</sub>, pH 7) and cultured in fresh medium containing different concentrations of doxycycline. Culture supernatants were collected 24 h later for further experiments. To concentrate the virus and eliminate Dox and soluble gp120, culture supernatants were centrifuged through  $1 \times 10^6$  MWCO columns (Sartorius) as specified by the supplier. Preliminary experiments using a constitutive expression vector for Env showed no inhibition of lentiviral production at any of the Dox concentrations used here.

#### *Flow cytometry analysis*

Assays were performed using the FACScan device from Becton Dickinson using  $10^4$  cells per sample. Env expression on 293 TetOn cells was analyzed as described (Lavillette et al., 1998) using the 2G12 human anti-gp120 antibody (Park et al., 2000) obtained from the NARRRP and a FITC-conjugated anti-human IgG1 antibody (Jackson ImmunoResearch Laboratories). As a negative control, cells were incubated with the secondary antibody alone. HR-TE virions-infected cells were scored through direct EGFP fluorescence analysis. NL4-3.HSA.R+E<sup>-</sup> virions-infected cells were identified after incubation at 4 °C for 30 min in the presence of the phycoerythrin (PE)-conjugated (M1/69) anti-mouse CD24 antibody (Becton Dickinson Pharmingen). Cells were washed in PBS before analysis. Cells incubated in the presence of the PE-conjugated anti-rat IgG2b were used as a negative control.

#### *Virus assay*

Dox on its own was shown not to influence infection. For Hos-CD4-CCR5<sup>hi</sup> cell cfu assay,  $1 \times 10^3$  cells were

plated per well in a 96-well culture plate (Nunc). Twenty-four hours later, the culture medium was replaced by fresh culture medium containing serially diluted culture supernatants or filtration-purified viruses in the presence of 8  $\mu$ g/ml polybrene (Sigma). EGFP-positive cells were scored under the fluorescence microscope 48-h post-infection and titers were calculated from the appropriate dilution. For FACS assays,  $10^4$  viral particles were used for infection. For Hos-CD4-CCR5<sup>hi</sup> cells,  $2 \times 10^4$  cells were plated in 12-well culture plates, infected for 24 h and FACS analysis was carried out 48 h post-infection. For macrophage infection,  $3 \times 10^5$  differentiated cells were infected in 12-well culture plates (Nunc). Infections were allowed to proceed for 16 h. The culture medium was changed and the number of infected cells was quantified 24 h later. Depending on the vector, either CD24 expression was determined by flow cytometry or EGFP fluorescence was measured by either flow cytometry or positive cells were directly counted under the fluorescence microscope. For PHA-activated PBL infection,  $2 \times 10^6$  cells per well of 12-well culture plates were placed in the presence of serial dilutions of viruses. Infections were allowed to proceed for 90 min at 30 °C under rotation at 2500 rpm. Culture plates were then transferred into an incubator and cells were washed twice with PBS 16 h later and once with RPMI medium. After 48 h in culture, they were analyzed by flow cytometry.

#### *Immunoblotting assays*

Viral and cell proteins were prepared and processed for immunoblotting as described elsewhere (Bachrach et al., 2000). For virion protein preparation, 1 ml virus-containing culture supernatant samples were adjusted to 10 mM CaCl<sub>2</sub> and left at 20 °C for 30 min. Precipitated viruses were spun down at  $15,000 \times g$  at 4 °C for 1 min and resuspended in 15  $\mu$ l of electrophoresis loading buffer. For cell protein preparation, cells were washed with PBS, scraped off culture dishes, centrifuged at  $1200 \times g$  at 4 °C for 5 min, resuspended in triplex lysis buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 0.2% NaN<sub>3</sub>, 0.1% SDS, 1% NP40, 0.5% Na deoxycholate, 2 mg/ml leupeptin, 1 mM phenylmethyl sulfone fluoride) and left on ice for 30 min. Cell debris and nuclei were removed by centrifugation ( $15,000 \times g$  at 4 °C for 10 min). One hundred micrograms cell protein or equal volumes of virus protein samples were processed for immunoblotting analysis. For Fig. 3B, 10  $\mu$ l of concentrated virions and 20  $\mu$ l of filtrates were used per track. For virion protein analysis, p24Gag was used as an internal reference to normalize data. gp120, gp41, and Gag proteins were detected using the DV-012 sheep polyclonal antibody, the 240-DM mouse monoclonal antibody and the SF2 rabbit monoclonal antibody, respectively, from the NARRRP. Densitometer analysis of appropriately exposed luminograms was performed using the NIH IMAGE software.

### Real-time PCR of provirus in infected cells

Seven days after infection with pNL4-3.HSA.R+E-HIV-1, genomic DNA from Hos-CD4-CCR5<sup>hi</sup> cells were extracted as described (Lin et al., 2002). In brief, cells were lysed in lysis buffer (10 mM Tris (pH 8), 0.5 mM EDTA, 0.0001% SDS, 0.001% Triton-X, 100 µg/ml Proteinase K) for 3 h at 50 °C, followed by 10 min at 95 °C and three freeze–thaw cycles. Debris was removed by centrifugation and quantitatively normalized DNA was amplified with oligonucleotides specific for the (1) Strong-Stop (5'-GCTCTCTGGCTAACTAGGGAAC-3' and 5'-TGAC-TAAAAGGGTCTGAGGGAT-3'), (2) the 5' LTR (GCTCTCTGGCTAACTAGGGAAC-3' and 5'-CTCTGGCTTTACTTTTCGCTTT -3') and (3) the CCR5 gene (5'-CGTCGACTCTCCCCGGGTGGAACAA-3' and 5'-TGGAT-CCAAGCCACAGATATTTCTGC-3') at 65 °C in a LightCycler (Roche Diagnostics) with SYBR green, as recommended by the manufacturer. To quantify the cellular DNA, the amplification was carried out so that the signal was obtained in the exponential range.

### NF-κB activation and luciferase assay

Hos-CD4-CCR5<sup>hi</sup> cells,  $2 \times 10^5$ , were seeded per well of 6-well culture plates and transfected 1 day later using the calcium phosphate coprecipitation procedure with 2 µg of the relevant firefly luciferase reporter plasmid and 0.1 µg of the Renilla luciferase expression plasmid (Promega) used as an internal transfection standard. Sixteen hours post-transfection, cells were washed three times with PBS and cultured for another 24 h, at which time they were subjected to infection for 6 h, washed again 3 times with PBS before replacing the culture medium. Firefly and Renilla luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega).

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## Host cell membrane proteins on human immunodeficiency virus type 1 after *in vitro* infection of H9 cells and blood mononuclear cells. An immuno-electron microscopic study

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Human immunodeficiency virus type 1 (HIV-1)-infected H9 and blood mononuclear cells (MNCs) were studied by immunogold electron microscopy for the presence of HIV-1 gag p24 protein, env gp41 and gp120 proteins, and host cell molecules CD4, CD11a, CD25, CD54, CD63, HLA class I and HLA-DR. Uninfected H9 cells and MNC membranes labelled for CD4, HLA class I and class II, and, at low density, CD11a and CD54; lysosomal structures in the cytoplasm labelled for CD63. The infected cell surface showed immunolabelling for HIV-1 proteins, as did budding particle-like structures. Immunogold labelling of the cell membrane for CD4 was almost non-existent. The level of immunolabelling

for CD11a and CD54 on infected cells was greater than that on uninfected cells; this is presumably related to a state of activation during virus synthesis. Budding particle-like structures and free virions in the intercellular space were immunogold-labelled for all host cell markers investigated. This was confirmed by double immunogold labelling using combinations of HIV-1 gag p24 labelling and labelling for the respective host cell molecule. We conclude that virions generated in HIV-1-infected cells concentrate host-derived molecules on their envelope. Also molecules with a prime function in cellular adhesion concentrate on the virion.

Infection of cells by human immunodeficiency virus type 1 (HIV-1) is followed by the disappearance of the virus receptor molecule CD4 from the cell membrane (Geleziunas *et al.*, 1991; Gielen *et al.*, 1989; Hoxie *et al.*, 1986). This phenomenon has also been observed for other surface molecules including HLA antigens (Eales *et al.*, 1988; Gelderblom *et al.*, 1987b; Henderson *et al.*, 1987; Kerkau *et al.*, 1989; Schols *et al.*, 1992) and the CD3, CD8 and CD11 antigens (Stevenson *et al.*, 1987). By using immuno-electron microscopy we have previously demonstrated the complete absence of CD4 antigen and the partial absence of HLA-DR and CD5 antigen on H9 cells 2 days after HIV-1 infection (Meerloo *et al.*, 1992). The CD3 and CD25 antigens remained detectable on the cell surface at similar density, and the CD63 antigen, a

lysosomal membrane glycoprotein, became detectable at higher density on cells after HIV-1 infection. In addition, CD3, CD4, CD5, CD25, CD30 and CD63 antigens, and HLA-DR are detected on budding figures and free virions in intercellular areas (Meerloo *et al.*, 1992). Thus, during the first phase after infection of H9 cells, host cell-derived molecules concentrate on newly generated HIV-1 virions. This phenomenon might contribute to the disappearance of these molecules from the cell membrane after infection. The present study focuses on the presence of adhesion molecules of the CD11a [ $\alpha$  chain of leukocyte function-associated antigen-1 (LFA-1)] and CD54 [intercellular adhesion molecule-1 (ICAM-1)] family on H9 cells after HIV-1 infection. In addition, we analysed blood mononuclear cells (MNCs) after *in vitro* infection with HIV-1 for the presence of host-derived molecules on budding particles and newly generated virions.

H9 cells were infected with HIV-1 IIIB strain 2 days before harvest by mixing one part infected cells with four

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Table 1. *Antibodies used in this study*

Antibody	CD*	Subclass	Source†	Reciprocal dilution‡	Reactivity
Anti-Leu-3a	CD4	G1	B&D	10	T helper cell phenotype
ADP 336	CD4	G2a	MRC	100	T helper cell phenotype
Anti-LFA-1 $\alpha$	CD11a	G1	AB	Undiluted	$\alpha$ -Chain of LFA-1
Anti-IL2R§	CD25	G1	B&D	10	Activated T cells
Anti-ICAM-1	CD54	G1	AB	12	ICAM-1
RUU-SP 5-15	CD63	G1	MM	3000	Platelets, macrophages and granulocytes
Anti-HLA-class I		Polyclonal	HP	300	Heavy chain of class I molecule
Anti-HLA-DR		G2a	B&D	10	HLA-DR
Anti-p24		Polyclonal	Seromed	2000	HIV-1 gag p24
10.15.64		G2a	Abbott	1000	HIV-1 env gp41
Anti-gp120		Polyclonal	Seromed	500	HIV-1 env gp120

\* CD, Cluster of differentiation.

† AB, A. C. Bloem, Utrecht, The Netherlands: CD11a, antibody F8.8 (Ahsmann *et al.*, 1992); CD54, antibodies F10.2 and F10.3 recognizing epitopes involved in cellular adhesion (Bloemen *et al.*, 1992). B&D, Becton Dickinson. HP, Professor H. Ploegh, Amsterdam, The Netherlands (Stam *et al.*, 1986). MRC, Medical Research Council, London, U.K. MM, M. Metzelaar, Utrecht, The Netherlands (Metzelaar *et al.*, 1991).

‡ Reciprocal dilution used in immunogold electron microscopy.

§ IL2R, Interleukin-2 receptor.

|| The sheep polyclonal antiserum was adsorbed to human tonsillar lymphocytes before use to avoid possible binding to human cellular constituents.

parts uninfected H9 cells. MNCs, isolated from heparinized blood from healthy donors by conventional Ficoll-Hypaque density gradient centrifugation and subjected to *in vitro* stimulation with phytohaemagglutinin in medium supplemented with polybrene and recombinant interleukin-2, were infected using a virus-containing cell-free supernatant of HIV-1 IIIB-infected H9 cells. Cells were harvested 3 weeks after infection, when virus production was maximal as determined by capture ELISA for HIV-1 gag p24 in the supernatant.

For conventional transmission electron microscopy (TEM), cell pellets were fixed in 2% glutaraldehyde (GA) in 0.1 M-cacodylate buffer pH 7.4, followed by embedding in Epon. Ultrathin sections were contrasted with uranyl magnesium acetate and Reynolds' lead citrate.

For immunogold labelling, cell pellets were fixed in 4% paraformaldehyde in 0.1 M-phosphate buffer pH 7.4 for 2 h at 4 °C, embedded in 10% gelatin and impregnated overnight at 4 °C with 2.3 M-sucrose. Ultrathin cryosections (80 nm) cut at -100 °C were subjected to the immunogold labelling procedure (Meerloo *et al.*, 1992). Post-sectioning immunogold labelling enables the detection of both intra- and extracellular determinants. The procedure included incubation with a first antibody (listed in Table 1), followed by a rabbit anti-mouse Ig antibody (diluted 1:400) or a rabbit anti-sheep Ig antibody (diluted 1:500) (Dakopatts) and finally with protein A-gold complex (15 nm gold particle size). Double immunogold labelling (Meerloo *et al.*, 1992) was done for HIV-1 gag p24 antigen (sheep antibody), and for HIV-1 env gp120 [using a monoclonal antibody (MAb)], HLA class I (rabbit antibody), HLA-DR (MAb), CD11a (MAb), CD54 (MAb) and CD63 antigen

(MAb). In this procedure, ultrathin cryosections were incubated with mouse or rabbit antibody, in the case of a mouse MAb followed by rabbit anti-mouse Ig. Sections were incubated with protein A-gold complex (15 nm particle size), and fixed for 10 min in 1% GA to prevent binding of the Protein-A gold complex used in the subsequent labelling. The subsequent incubation was done with sheep anti-HIV-1 p24 antibody followed by rabbit anti-sheep antibody and Protein A-gold complex (10 nm particle size). The sections were embedded and contrasted in 1.8% methylcellulose containing 3% uranyl acetate pH 7.0. The sections were examined in a Jeol 1200 EX electron microscope.

The optimal dilution for each antibody was determined in preliminary titration experiments. Controls included incubation of the anti-HIV-1 antibody with uninfected H9 cells or uninfected MNCs. In addition, primary or secondary antibodies were omitted or applied at higher dilutions in single or double labelling experiments. To exclude labelling as a result of non-specific Ig isotype binding to virus particles, we incubated H9 cells with MAbs anti-Leu-5b (IgG2a, CD2) and anti-Leu-2a (IgG1, CD8), or a polyclonal rabbit anti-fluorescein isothiocyanate antiserum, which did not label cells. Labelling was not observed in any control experiment.

The preparations of blood MNCs after culture showed a mixed cell population including lymphoblastoid cells and macrophages. Conventional TEM of infected H9 cells and MNCs revealed free virions and virions attached to the cell surface, with ultrastructural features similar to those described previously (Meerloo *et al.*, 1992; Gelderblom *et al.*, 1987a; Hausmann *et al.*, 1987; Marx *et al.*, 1988; Palmer & Goldsmith, 1988; Timár *et al.*, 1986).



Fig. 1. Immunogold labelling of MNCs for HIV-1 p24. Although there is some immunogold label in the cytoplasm, most localizes to the cell membrane, on structures resembling budding particles. Virions attached to the cell membrane are also labelled. Immunolabelling can be seen on the cell membrane separated from virions (arrowhead). Bar marker represents 200 nm.

Budding particles were observed on about 80% of H9 cells and about 5% of cells in MNC preparations. The incidence of budding particles on H9 cells 2 days post-infection was higher than that on chronically infected cells (about 40%), indicating that budding figures are derived from newly infected cells. In cryosections of infected MNCs the typical characteristics of virions were lost, but virions could be identified easily by immunogold labelling for HIV-1 antigens. Free virions were labelled, as well as structures resembling budding particles (Fig. 1). The anti-p24 antibodies labelled a product located mainly in the core of virions and budding particle-like structures. The immunogold labelling for env antigens gp41 and gp120 was localized more to the membrane part of the virus. Immunolabelling for viral antigens was also observed scattered in the membrane region and in the cytoplasm of cells (Fig. 1). The density of p24 labelling was greater than that for gp41 or gp120 (data not shown). These data were similar for H9 cells and MNCs (Fig. 1), but the number of immunolabelled cells in preparations of MNCs was much lower (about 5%).

Immunogold labelling for HLA class I and HLA-DR was found on the membrane of uninfected H9 cells and MNCs; labelling was also observed in the cytoplasm of

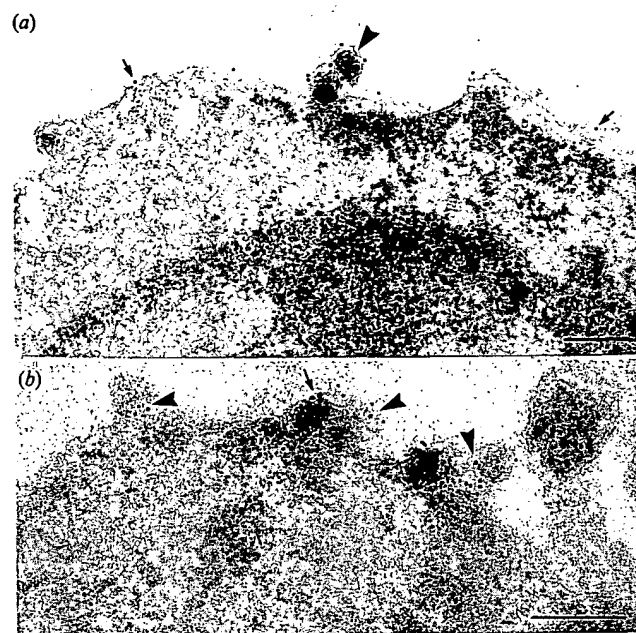


Fig. 2. (a) Immunolabelling of an HIV-1-infected MNC for HLA-DR. Label can be seen on virus-like particles at the cell membrane (arrowhead) and on the cell membrane separated from virus-like structures (some indicated by arrows). Bar marker represents 200 nm. (b) Double immunogold labelling of HIV-1-infected MNC with CD4 antibody (15 nm gold particles, arrow) and antibody to HIV-1 p24 (10 nm gold particles, arrowheads). The CD4 immunogold label is present on a virus-like structure, but is not separated from such structures on the cell membrane. Labelling for p24 can be seen on virus-like particles and on the cell membrane. Bar marker represents 200 nm.

the cells. On infected H9 cells and MNCs (Fig. 2a), the density of labelling on the cell surface was somewhat lower. Labelling was observed on budding particle-like structures and free virions in the intercellular space. In double immunogold labelling experiments, co-localization of HLA class I (Fig. 3a) or HLA-DR label and HIV-1 p24 label was observed on virus particles and budding particle-like structures. In the cytoplasm, HLA class I (Fig. 3a) or II immunolabelling segregated from that for p24. The preservation of cytoplasmic components was such that it was not possible to identify which structures were labelled by either anti-p24 or anti-HLA antibody.

Immunogold labelling for CD4 was readily visible on the membrane of uninfected cells. This was observed for port of MNCs and for H9 cells. Infected cells, either MNCs or H9 cells, showed no CD4 immunogold labelling of the cell membrane. Immunolabelling was observed on budding particle-like structures and on free virions in the intercellular space. This was confirmed by double labelling for CD4 and p24 (Fig. 2b). The data were similar for antibodies anti-Leu-3A and ADP 336.

CD25 cell surface immunolabel was observed on H9 cells, and at a higher density on some MNCs. Infected



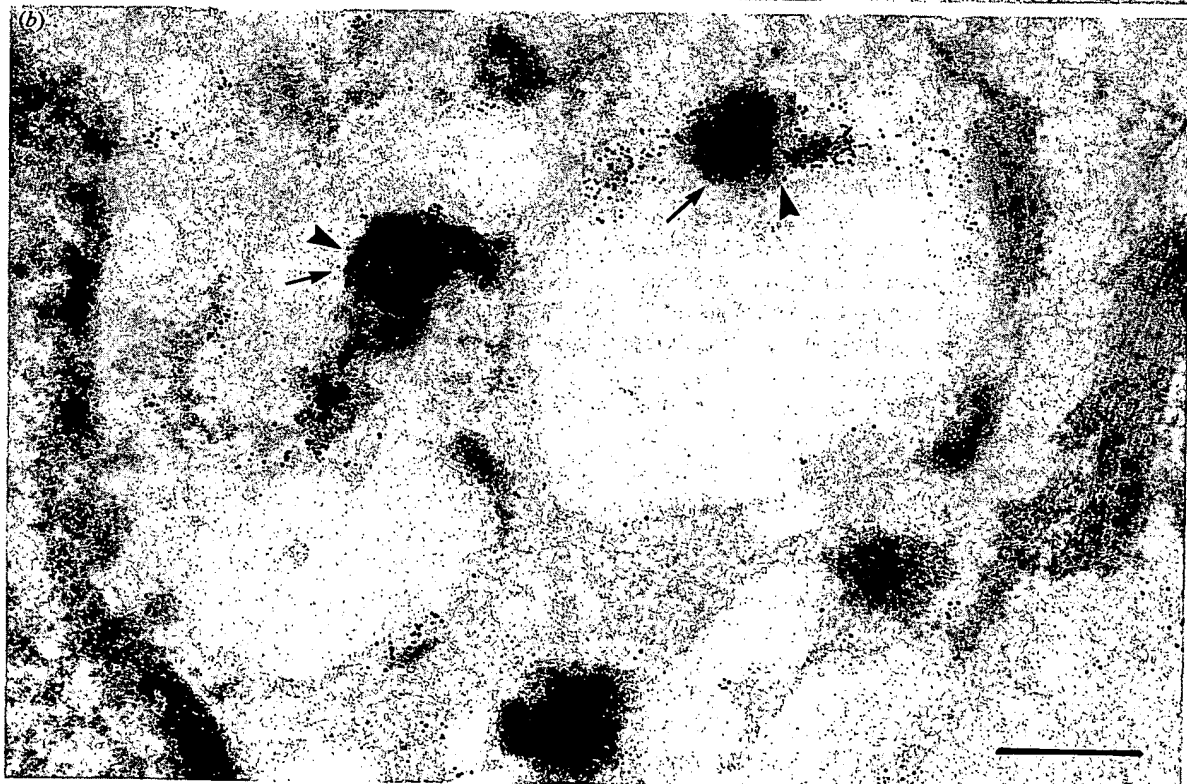
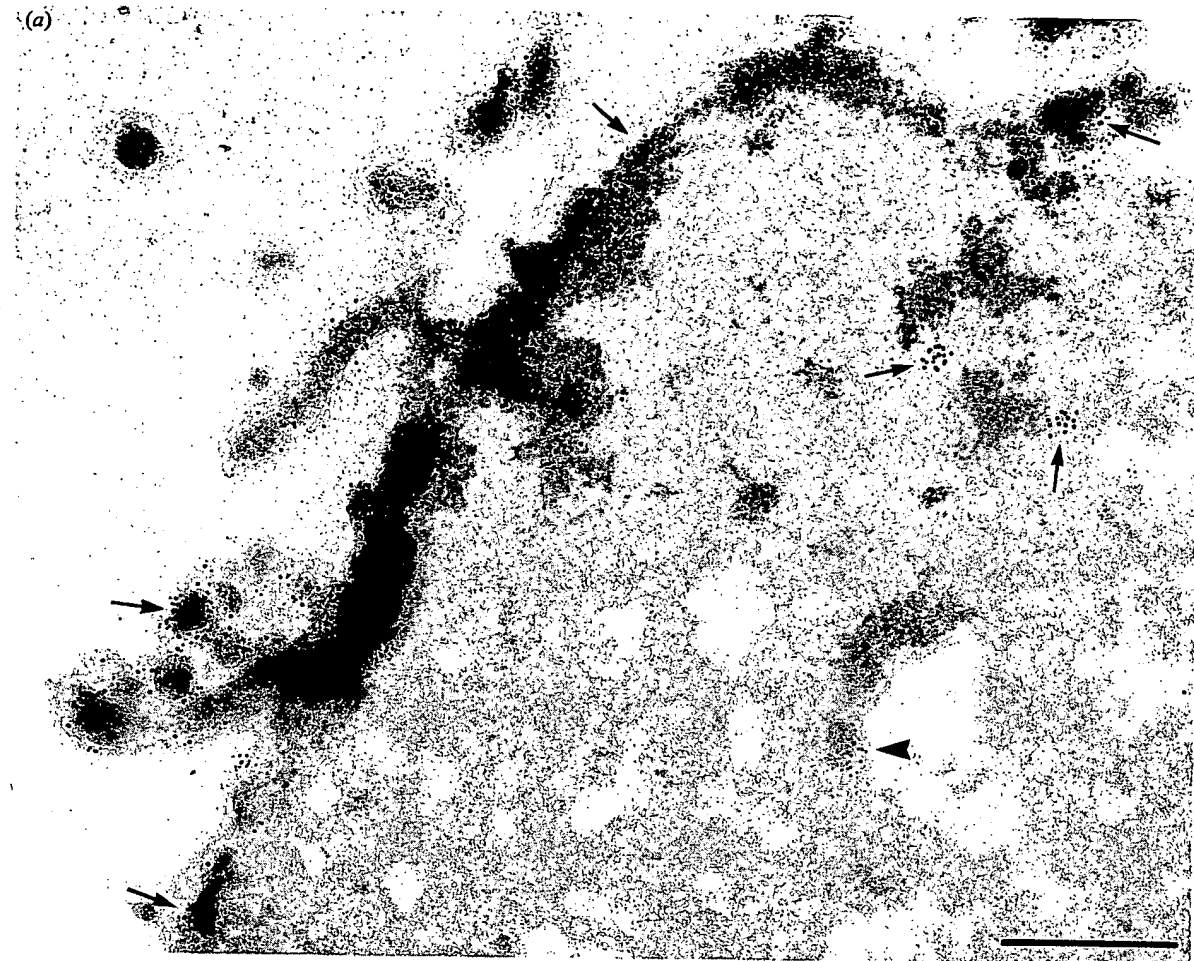






Fig. 3. Double immunogold labelling of HIV-1-infected H9 cells with anti-HLA class I (a) or CD63 antibody (b, c) using 15 nm gold particles, and anti-HIV-1 p24 using 10 nm gold particles. (a) The immunogold labelling for HLA class I (arrows) is localized to the cell membrane and virus-like particles together with HIV-1 p24 (arrowhead). In the cytoplasm, the HLA class I immunolabel localizes separately from the HIV-1 p24 label. It was not possible to identify which cytoplasmic structure was labelled by either the anti-p24 or the anti-HLA antibody. Bar marker represents 500 nm. (b, c) Labelling for CD63 (arrows) co-localizes with that for HIV-1 p24 (arrowheads) in lysosomal structures in the cytoplasm (b) and on virus-like particles in the extracellular area (c). Bar markers represent (b) 500 nm, (c) 200 nm.

cells showed CD25 immunogold label on budding particle-like figures and virus particles in the intercellular space (data not shown). The CD63 antibody labelled cytoplasmic structures in uninfected H9 cells and some MNCs. The label was associated with lysosomal and vesicular structures in the cytoplasm. On infected H9 cells and MNCs, CD63 immunogold labelling of budding particle-like structures and free virions was seen. In double immunogold labelling experiments, the CD63 and HIV-1 p24 label co-localized to the same site in the cytoplasm (Fig. 3b), presumably in lysosomal structures. Co-localization on virions was also observed (Fig. 3c).

In immunogold labelling experiments for CD11a (Fig. 4a, b, c) and CD54 (Fig. 4d), the CD54 reagents F10.2 and F10.3 gave similar results. Uninfected H9 cells showed only a low density of CD11a on the cell membrane (Fig. 4a). Similarly, there was low density immunolabelling by anti-CD54 antibodies. On infected H9 cells the density of CD11a or CD54 was greater. This observation confirms the data of Weeks *et al.* (1991) who documented enhanced expression of  $\alpha_5\beta_1$  integrin on T lymphocytes after HIV-1 infection. Presumably this is related to a state of activation of the cells in the first period after infection. The CD11a and CD54 labelling also localized to budding particle-like figures and free virions in the intercellular space (illustrated for CD11a in Fig. 4b, for CD54 in Fig. 4d). This was confirmed by double labelling experiments with a combination of anti-p24 antibody and either an anti-CD11a (Fig. 4c) or CD54 antibody. In preparations of MNCs only a few cells were immunolabelled by the anti-CD11a and anti-CD54 antibodies. Virion structures on these cells were

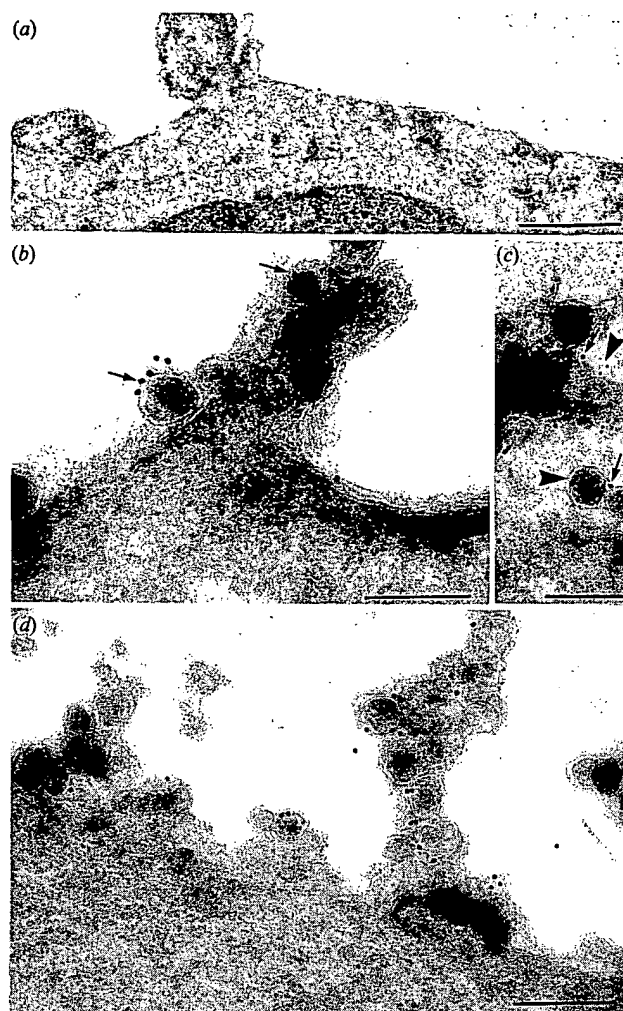


Fig. 4. (a, b) Immunogold labelling for CD11a (LFA-1  $\alpha$ -chain) of uninfected (a) and HIV-1-infected (b) H9 cells. Uninfected H9 cells show CD11a label on the cell membrane. On HIV-1-infected cells, label is observed on virus-like particles (arrows) and on the cell membrane. Bar markers represent (a) 500 nm, (b) 200 nm. (c) Double immunogold labelling for CD11a (15 nm particle size, arrows) and HIV-1 p24 (10 nm particle size, arrowheads), showing co-localization of CD11a and HIV-1 p24 to virus-like particles. Bar marker represents 200 nm. (d) Immunogold labelling of HIV-1-infected H9 cells for CD54 (ICAM-1), showing localization on virus-like particles adjacent to cells. Bar marker represents 200 nm.

also labelled; this labelling co-localized with HIV-1 p24 immunolabelling.

The presence of host-derived molecules on newly generated virions has been demonstrated for CD3, CD4, CD5, CD25, CD63 (Fig. 3c), HLA-DR and HLA class I (Fig. 3a) (Gelderblom *et al.*, 1987b; Henderson *et al.*, 1987; Kerkau *et al.*, 1989; Meerloo *et al.*, 1992; Schols *et al.*, 1992). Both H9 cells and blood MNCs after *in vitro* infection show host-derived molecules on budding particle-like figures and virions in the intercellular spaces (Fig. 2). From these results we conclude that the uptake

of host-derived molecules by forming virions may be a general phenomenon in HIV-1-infected cells actively producing virus. In addition, this study has demonstrated that virions carry adhesion molecules LFA-1 and ICAM-1 (Fig. 4*b, c, d*), that appear to be up-regulated on the surface of H9 cells after infection. There is no apparent selectivity in the insertion of host-derived cell surface molecules to concentrate on virions during the budding process. This conclusion contrasts with the previous results showing non-random association, e.g. for HLA-DR but not HLA-DP and HLA-DQ using flow cytometry (Schols *et al.*, 1992), and for HLA-class I and II and  $\beta_2$ -microglobulin, but not 11 other cell surface components, using ELISA of solubilized virions (Hoxie *et al.*, 1987). Selectivity in the presence of certain host-derived molecules on vesicular stomatitis virus (VSV) and murine leukemia virus (MLV) (described below) has also been described (Calafat *et al.*, 1983). This discrepancy can be ascribed to differences in the methods applied. Quantitative data on the number of host-derived molecules on virions, in relation to that on the host cell membrane, may give additional information on this discrepancy, but immunoelectron microscopy is not directly suitable for such measurements.

The relevance of the presence of host-derived molecules on newly synthesized virions should be considered while bearing in mind that cells were infected *in vitro*. To investigate this phenomenon in *in vivo* infection, we examined blood MNCs from HIV-1-infected patients after *in vitro* stimulation with mitogens. This approach was not successful; the proportion of cells actively producing virus, as judged by examining budding structures, was too low to make reliable observations (data not shown).

The presence of host-derived molecules on virions is not unique for HIV-1. It has also been documented for Friend leukaemia virus (Chen & Lilly, 1979), avian leukosis virus (Young *et al.*, 1990), VSV (Hecht & Summers, 1976; Calafat *et al.*, 1983), MLV (Calafat *et al.*, 1983), Sindbis virus (Strauss, 1978) and influenza virus (Holland & Kiehn, 1970). Conversely, Simons & Garoff (1980) have shown that budding structures of Semliki Forest virus do not contain host-derived molecules. The relevance of the presence of host-derived molecules remains subject to speculation. Immunoelectron microscopy does not distinguish between proteins inserted into the envelope membrane of the virus and proteins attached to the viral envelope.

Our findings may have implications for the interaction between virions and cells. The virus may use host-derived molecules in addition to env protein in binding and subsequent infection of other (CD4-negative) cells, and in this way contribute to the spread of infection. This phenomenon is particularly relevant for cellular adhesion

molecules, which have a prime function in intercellular contacts (Dustin & Springer, 1991). In this study, the interaction between ICAM-1 and LFA-1, which mediate leukocyte adhesion and signalling, has been examined. The CD54 antibodies used (F10.2 and F10.3) recognize epitopes involved in cellular adhesion (Bloemen *et al.*, 1992). The LFA-1 molecule has been shown to be involved in *in vitro* syncytium formation by HIV-1-infected cells (Hildreth & Orentas, 1989; Valentin *et al.*, 1990), but not in HIV spread and virus replication (Pantaleo *et al.*, 1991).

This use of host-derived molecules adds to the range of potential mechanisms by which the cell binds HIV-1 and may subsequently become infected. These mechanisms include the CD4-env gp120 interaction (Meltzer *et al.*, 1990; Tersmette & Miedema, 1990) and interactions between HIV-1-containing complexes and the receptor for the Fc part of Ig (Homsy *et al.*, 1989; Takeda *et al.*, 1988) and that for the C3 component of complement (June *et al.*, 1991). When designing *in vivo* therapies to prevent adherence and spread of HIV-1 infection in the body, one should be aware of the possibility that the virus may not only use its own envelope components, but also may use host-derived adhesion molecules in binding and subsequent infection.

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## Immunohistochemical detection of HIV structural proteins and distribution of T-lymphocytes and Langerhans cells in the oral mucosa of HIV infected patients\*

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**Summary:** Seventeen biopsies taken from oral mucosa of HIV infected patients were analysed for the distribution of CD4<sup>+</sup>/CD8<sup>+</sup> T-lymphocytes and Langerhans cells. The results were evaluated statistically. An increase in the absolute number of CD4<sup>+</sup> and CD8<sup>+</sup> cells in HIV infected patients without clinical symptoms, ARC or AIDS was seen in the connective tissue stroma when compared with normal oral mucosa from sero-negative patients. However, the ratio between CD4<sup>+</sup>/CD8<sup>+</sup> cells was decreased due to the disproportionate increase of CD8<sup>+</sup> cells. These findings did not show statistical correlation with the clinical status of the infection. In contrast to the increase in absolute number of CD4<sup>+</sup>/CD8<sup>+</sup> cells the number of Langerhans cells was unchanged when compared with the control group. Using four different monoclonal antibodies against virus structural proteins (2 × anti p24, gp41, gp120) two of 26 patients showed labelled cells in consecutive sections in the same connective tissue areas. It was assumed that latently infected mononuclear cells invaded the oral mucosa together with uninfected cells of the cellular immune system.

**Key words:** AIDS – HIV – Langerhans cells

### Introduction

The human immunodeficiency virus (HIV), a member of the lentivirinae subfamily of the retrovirus family, is the aetiological agent of the acquired immunodeficiency syndrome (AIDS) and related disorders. Soon after the isolation of HIV, the ge-

nome was sequenced (Chiu et al. 1985; Sonigo et al. 1985) and virus structural proteins were characterized (for a review see: Wong-Staal and Gallo 1985). It was shown that the virus envelope glycoprotein gp 120 of HIV binds specifically to epitopes of the CD4-receptor molecule (Dalglish et al. 1984; Klatzmann et al. 1984). The interaction between gp 120 and the cell receptor has been considered to be a necessary and critical step for initiating the reproductive cycle of HIV in its host cell (McDougal et al. 1986).

Clinically, AIDS is characterized by multiple opportunistic infections and/or malignancies, predominantly of the Kaposi sarcoma (KS) type (Friedman-Kien et al. 1982; Reichart et al. 1987). The AIDS-related complex (ARC) encompasses milder forms and sometimes prodromal states of the disease, that is unexplained chronic lymphadenopathy and/or lymphopenia involving CD4<sup>+</sup> T-lymphocytes (Masur et al. 1981).

HIV can regularly be isolated from CD4<sup>+</sup> lymphocytes of infected persons. However, HIV can obviously replicate in activated B-lymphocytes in addition and in monocyte – macrophage cells, in Langerhans cells (LC) of the skin, in follicular dendritic cells of infected lymph nodes and in brain cells of the monocyte – macrophage or microglia lineage (Montagnier et al. 1984; Levy et al. 1985; Shaw et al. 1985; Gartner et al. 1986; Tenner-Racz et al. 1986; Tschachler et al. 1987). All these cells express low densities of the CD4 surface marker (Wood et al. 1984; Levy et al. 1985; Stewart et al. 1986), which was thought initially to be exclusive to helper lymphocytes (Reinherz et al. 1979).

The oral mucosa contains numerous cells of the immune system in both the epithelium and in the connective tissue. In the epithelium two main groups of immunocompetent cells are present, bone marrow-derived dendritic cells, LC, and T-

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Table 1. Antibodies used

Designation	Reactivity	Reference	Dilution
CD4	Inducer/helper T-lymphocytes	Coulter Immunology, Florida, USA	1:1000*
CD8	Suppressor/cytotoxic T-lymphocytes	Coulter Immunology, Florida, USA	1:1000*
CD1	Seventy per cent of thymocytes, Langerhans cells	Coulter Immunology, Florida, USA	1:1000*
p24	HIV core protein	Dr. R.C. Gallo, NIH, Bethesda, USA	1:10000
p24	HIV core protein	Dr. H. Lutz, Veterinär-Medizinische Klinik Zürich, Switzerland	1:5000
gp41	HIV transmembranous protein	Dr. L. Montagnier, Pasteur Institute, Paris, France	1:200
gp120	HIV envelope protein	Dr. L. Montagnier, Pasteur Institute, Paris, France	1:200

\* 50 test vial

lymphocytes. In the connective tissue T-lymphocytes, cells of the macrophage system and B-lymphocytes predominate (Becker et al. 1985). In the mucosa and skin LC form a reticular epithelial trap for external antigens and act as antigen-presenting cells for T-lymphocytes. It is well documented that LC carry antigens identical to those of classical macrophages, like HLA-DR (Nagy et al. 1986). Furthermore, they express low densities of the CD4 receptor. Due to the presence of the CD1 antigen they can be differentiated from these macrophages (Murphy et al. 1981). Numerous studies have been performed on the distribution of T-lymphocyte subsets in peripheral blood of ARC and AIDS patients (Weber et al. 1986), but only few studies have focussed on immunopathological changes in skin and oral mucosa during the course of HIV infection. Because intense quantitative phenotype alterations within the skin LC population of ARC/AIDS patients were described by Belsito et al. (1984) the distribution of LC and of T-lymphocyte subsets was studied in the oral mucosa of HIV infected patients to get further insight in the local immune system of the oral mucosa during HIV infection.

Since HIV has recently been demonstrated in skin LC (Tschachler et al. 1987) we attempted to demonstrate the presence of HIV in the oral mucosa using monoclonal antibodies against different virus structural proteins.

## Material and methods

For the investigation of CD4<sup>+</sup>- and CD8<sup>+</sup>-T-lymphocytes and Langerhans cells 17 patients were divided into three clinical groups, according to the definition of the Centers for Disease Control (1986). The first was those who were HIV infected, clinically without any signs of infection or oral candidiasis ( $n=5$ ), the second ARC ( $n=6$ ) and the third AIDS ( $n=6$ ). HIV infection was diagnosed by ELISA and Western blot. 17 biopsies were taken under local anaesthesia from clinically normal oral mucosa (buccal mucosa: 2, gingiva propria: 9, tongue: 4, hard palate 2), snap frozen in liquid nitrogen and stored at  $-75^{\circ}\text{C}$ . All AIDS patients revealed oral candidiasis. In these

patients the ratio between CD4<sup>+</sup>/CD8<sup>+</sup> cells of peripheral blood lymphocytes was determined by flow cytometry using fluorescein-conjugated monoclonal antibodies (Coulter Immunology) and ranged between 0.04–0.4.

For the detection of HIV virus structural proteins (Table 1) biopsies of 26 patients were examined (1. HIV infected,  $n=7$ , 2. ARC,  $n=8$ , 3. AIDS,  $n=11$ ). 18 biopsies were taken from oral mucosa, 5 biopsies from oral KS (hard palate) and 3 biopsies from oral hairy leukoplakia (tongue). As a control 10 biopsies of normal oral mucosa of non-HIV infected patients were included (gingiva propria,  $n=7$ , tongue  $n=3$ ). Two patients were females (control: 4) and 24 were males (control: 6). The average age of the three clinical groups was 34 years (range 24–59 years), that of the control group 20 years (range 10–33 years).

Consecutive cryostat sections of 3–4  $\mu\text{m}$  in thickness were air dried for 2 h and fixed in acetone for 15 min at room temperature. Sections were first incubated with primary monoclonal antibodies (Table 1), then with the unlabelled goat-antimouse bridging antibody (dilution 1:60; Jackson, Avondale, USA) and finally with the alkaline phosphatase – mouse-anti-alkaline phosphatase (APAAP) immune complexes (dilution 1:50; Dianova, Hamburg, FRG). For reference see Cordell et al. (1984). Monoclonal antibodies were diluted with bovine serum albumin (1%) in tris buffered saline (TBS, 0.05 M, pH 7.5). At all steps, sections were incubated for 30 min. The second and third step incubations were each repeated twice for 10 min. After every incubation sections were washed in TBS buffer for 3  $\times$  5 min. The alkaline phosphatase substrate was prepared as follows: 0.5 ml of a 5% solution of sodium nitrite was added to a solution of 170 ml Tris-HCL (0.05 M, pH 8.7) and 60 ml 0.2 M aminomethylpropanediol containing 90 ml of levamisole, followed by the addition of 125 mg of naphthol AS-BI which had been freshly dissolved in dimethylformamide at 10 mg/ml. This solution was then filtered and used immediately for the staining of slides (15–30 min). The slides were counterstained with haematoxylin for 1–3 min.

Control reactions were explored for each antibody and biopsy by omitting the primary antibody. Monoclonal antibodies against virus structural proteins were tested for specificity on the H9 and HTLV-IIIb infected H9 T-cell line (Fig. 1) and on tonsillar tissue of HIV sero-negative persons.

The commercially available bridging antibody led to a non-specific background staining in the entire connective tissue. This was probably due to circulating immune complexes, not marked in the control group but pronounced in HIV infected patients. The non-specific reactivity was reduced as follows: 50  $\mu\text{g}$  of a biopsy of a HIV patient, which revealed a pronounced background staining, was homogenized in 1 ml PBS containing 0.02% sodium azide, sedimented by centrifugation and the residue was washed three times in the same buffer. 0.5 ml of the

Table 2. Quantitation of labelled cells (mean values  $\pm$  SD)

		CD4 <sup>+</sup>	CD8 <sup>+</sup>	CD1 <sup>+</sup>
Normal oral mucosa (n=10)	E	10,6 $\pm$ 5,2	6,3 $\pm$ 3,0	8,9 $\pm$ 5,2
	C	17,7 $\pm$ 11,1	7,6 $\pm$ 4,6	0,7 $\pm$ 1,0
I. HIV infected (n=5)	E	7,5 $\pm$ 5,6	14,8 $\pm$ 14,0	8,3 $\pm$ 3,9
	C	49,8 $\pm$ 23,0	65,1 $\pm$ 28,8	0,6 $\pm$ 0,9
II. ARC (n=6)	E	6,7 $\pm$ 6,7	19,0 $\pm$ 9,1	7,6 $\pm$ 5,0
	C	25,3 $\pm$ 19,3	28,4 $\pm$ 14,8	2,1 $\pm$ 1,1
III. AIDS (n=6)	E	6,4 $\pm$ 3,8	10,5 $\pm$ 4,7	4,1 $\pm$ 2,7
	C	40,1 $\pm$ 14,7	27,6 $\pm$ 26,5	0,8 $\pm$ 1,2

E = epithelium; C = connective tissue; n = number of biopsies

original bridging antibody was diluted 1:2 and incubated with the resuspended tissue pellet for 4 h at 4°C by vigorous shaking.

This procedure led to a nearly complete reduction of the unspecific background staining in the connective tissue but not to a reduction of the specific staining. The same dilution of the bridging antibody was used as before.

The epithelium and upper connective tissue stroma were examined separately. In each, 10 randomly distributed fields were evaluated using a Leitz Orthoplan microscope with a primary magnification of  $\times 400$ . The mean values of the counted cells in ten fields were calculated. Only those cells with clearly recognizable nuclei were counted. For each of the four groups (Table 2) the mean value of each cell type (CD1<sup>+</sup>, CD4<sup>+</sup> and CD8<sup>+</sup>) with its standard deviation (SD) was calculated. To decide whether there was a significant difference between the groups the values were examined using an analysis of variance (significance level 5%). The number of CD1<sup>+</sup>, CD4<sup>+</sup> or CD8<sup>+</sup> positive cells in the normal oral mucosa of the HIV seronegative control group were compared with the number of positive cells of the oral mucosa of all 3 groups of HIV infected patients, also the 3 individual groups of HIV infected patients were compared against each another.

The mean value and standard deviation (SD) of the ratio between CD4<sup>+</sup> and CD8<sup>+</sup> cells in the epithelium and in the connective tissue was determined.

## Results

The mean values and the SD of the different immunocompetent cells in the four groups investigated are summarized in Table 2. Using an analysis of variance the quantitative increase of CD4<sup>+</sup> and CD8<sup>+</sup> positive cells in the connective tissue and the increase of CD8<sup>+</sup> positive cells in the epithelium of the groups with HIV infection differed significantly compared with the normal oral mucosa of the control group. In contrast, the number of CD4<sup>+</sup> cells was decreased in the epithelium of all HIV infected patients (Fig. 2). Statistically, there was no significant difference in the number of CD4<sup>+</sup> and CD8<sup>+</sup> positive cells between the individual three groups of HIV infected patients although the pattern of

distribution was different. In the first two groups, T-lymphocytes were found predominantly in the upper connective tissue and in the epithelium (CD8<sup>+</sup> cells; Fig. 3) and often aggregations of labelled cells were observed. In AIDS patients, however, the labelled cells were often more diffusively distributed in the upper but also in the deeper connective tissue stroma. The ratio between CD4<sup>+</sup> and CD8<sup>+</sup> cells is shown in Table 3. The mean values and the SD indicated a decreased ratio in the connective tissue of HIV infected patients. This was due to the relatively greater increase of CD8<sup>+</sup> cells when compared with CD4<sup>+</sup> cells (Table 2).

CD1<sup>+</sup> cells were observed only in the upper connective tissue close to basement membrane and within the epithelium itself. Statistically, there was no significant difference in the values of LC between normal oral mucosa and HIV infected patients and between the three individual groups of HIV infected patients itself.

In two out of 26 biopsies of HIV infected patients, labelling for HIV structural proteins was detected. Consecutive sections showed labelled cells in the same connective tissue area positive for all HIV-specific monoclonal antibodies. All antibodies revealed a similar staining pattern. Positive cells could not be detected in the epithelium. One patient revealed brilliantly labelled mononuclear cells in the specific but also in the control reaction in one area of the deeper gingival connective tissue stroma. All other biopsies were negative. The first positive biopsy (Fig. 4) was taken from clinically normal gingiva from a 35-year-old female drug abuser, who developed ARC 4 weeks after biopsy. A second biopsy was taken from an oral hairy leukoplakia (tongue) of a 45-year-old homosexual man. The quantity and staining intensity of labelled cells was more pronounced in the first biopsy, in the second biopsy staining intensity of only few cells was comparable to the first patient (Fig. 5). Neither a higher concentration of primary monoclonal antibodies nor using a mixture of three different antibodies (anti p24, gp41, gp110) resulted in an increase of staining intensity.

## Discussion

All covering epithelia and their subepithelial connective tissue stroma contain numerous cells of the immune system. A number of studies have been performed on the distribution of immunocompetent cells in various diseases of the oral mucosa (Becker et al. 1983, 1985). The ratios of T-helper/inducer (CD4<sup>+</sup>) and T-suppressor/cytotoxic (CD8<sup>+</sup>) T-lymphocytes in normal oral mucosa in

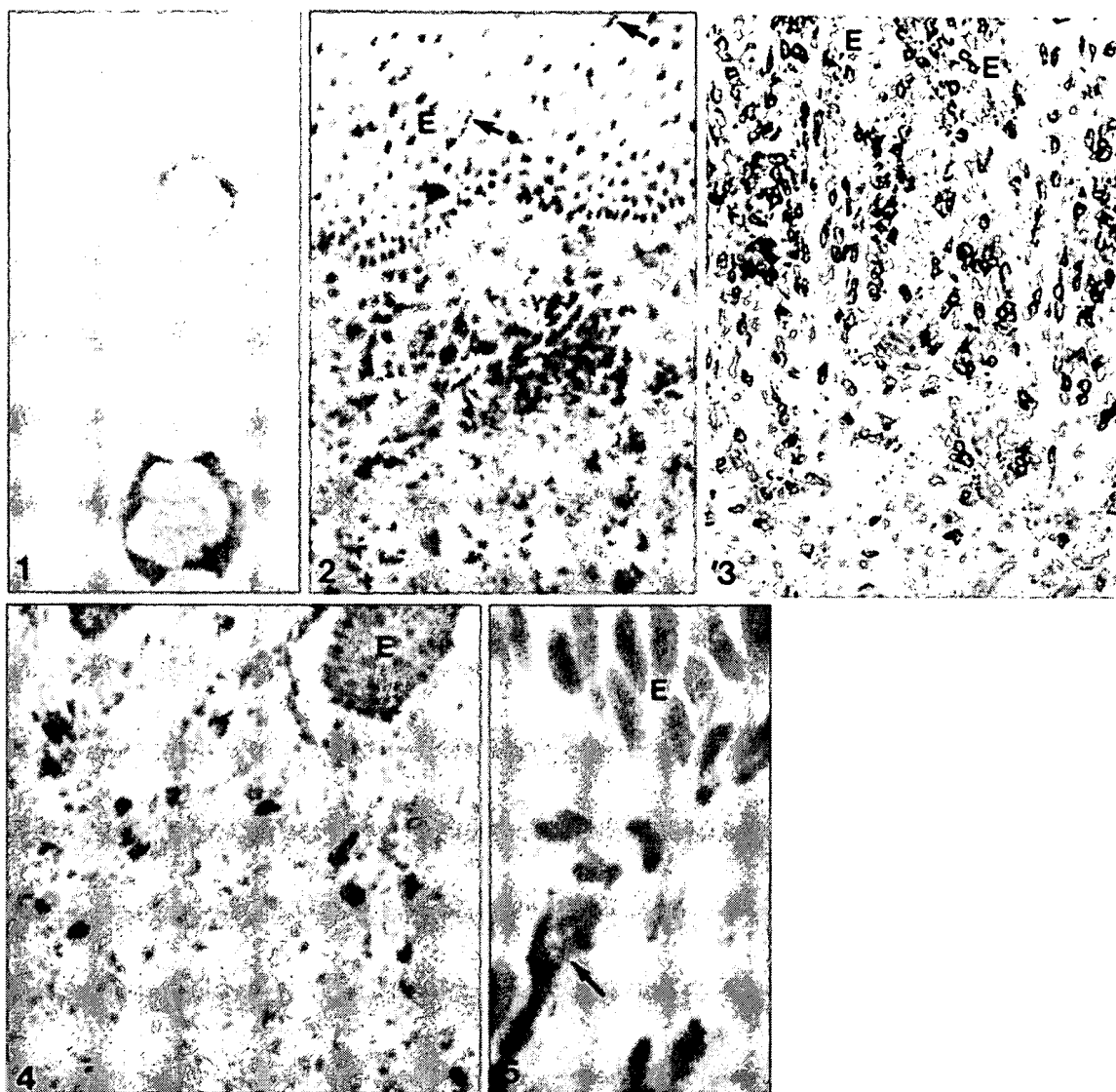


Fig. 1. H9-HTLV IIIB infected H9T-cell line: Positive staining for p24.  $\times 1900$

Fig. 2. Numerous  $CD4^+$  cells in the connective tissue of an AIDS patient, often forming aggregations of lymphocytes. In the epithelium (E) positive cells were rarely observed.  $\times 1200$

Fig. 3. Numerous  $CD8^+$  positive cells in the epithelium (E) and in the connective tissue (same patient as Fig. 3).  $\times 800$

Fig. 4. Normal gingiva propria of an HIV infected patient with numerous gp41 positive cells in the connective tissue stroma.  $\times 825$

Fig. 5. One cell positive for p24 (arrow) in the upper connective tissue of a hairy leukoplakia. (E=epithelium)  $\times 3000$

these studies differed when compared to our present data. In this study the ratio of  $CD4^+$  and  $CD8^+$  cells in the epithelium was 1.76 and 2.53 in the connective tissue. In a previous study (Becker et al. 1985) the ratio was 0.51 in the epithelium and balanced in the connective tissue stroma. These differences might be due to different staining

techniques. While in former studies usually the immunoperoxidase technique was used, in the present investigation the APAAP technique was applied. To decide whether different primary monoclonal antibodies with different avidity and/or recognition of epitopes for the  $CD4$ -molecule label different quantities of  $CD4$ -positive cells in peripheral



**Table 3.** Ratio between CD4<sup>+</sup> and CD8<sup>+</sup> cells in the oral mucosa (mean values and SD)

		x	SD
Normal oral mucosa (n=10)	E	1,76±0,69	
	C	2,53±1,37	
I. HIV infected (n=5)	E	1,06±0,87	
	C	0,85±0,37	
II. ARC (n=6)	E	0,36±0,29	
	C	0,82±0,21	
III. AIDS (n=6)	E	0,69±0,44	
	C	1,82±1,39	

tissues would require further double labelling studies. Furthermore, differences in cell distribution, especially in the absolute number of immunocompetent cells, could be related to sampling of different regions of the oral mucosa, as it was shown for Langerhans cells by Daniels et al. (1984). The quantitative increase of labelled CD4- and CD8-positive cells in the connective tissue was found in all locations where biopsies have been taken. This may indicate that the connective tissue stroma of oral mucosa reveals an increased lymphocytic infiltration during the HIV infection. The intense, diffuse infiltration of the deeper connective tissue was only observed in AIDS patients and has not been found in other oral diseases, i.e. oral papillomas, leukoplakias or lichen planus (Becker et al. 1983; 1985). These findings were in contrast to the results found in peripheral blood. While in peripheral blood a continuous decrease of CD4<sup>+</sup> cells was observed, in the connective tissue of the oral mucosa the absolute number of CD4- and CD8-positive cells increased. This decreased ratio was obviously due to an disproportional increase of CD8<sup>+</sup> cells, but reflects also the decrease of CD4<sup>+</sup> cells in peripheral blood.

LC take up foreign antigens and present them together with their own HLA-DR, probably to the CD4 receptor bearing T-lymphocytes (Stewart et al. 1986). In a previous study of oral papillomas and leukoplakias a simultaneous increase of T-lymphocytes and LC was found (Becker et al. 1985). In contrast to these findings the present study revealed an unchanged number of LC in HIV infected/ARC and AIDS patients compared with the control group, although an increase of LC was expected from our previous studies (Becker et al. 1985). Belsito et al. (1984) and Oxholm et al. (1986) observed a reduction of Ia-positive LC in skin biopsies of ARC/AIDS patients and it was suggested that a reduced capacity for Ia antigen and thereby antigen presentation might result in

a defective CD4<sup>+</sup> helper cell proliferation. This might therefore play a role in the pathogenesis of HIV infection (Belsito et al. 1984). This theory was underlined by the findings of Tschachler et al. (1987), demonstrating that epidermal LC are a target for HIV and that on the electron microscopic level signs of cellular alterations of LC seemed to be due to HIV particles within LC. The unchanged number of LC in the present study, despite the inflammation, may also indicate reduced antigen presentation, but labelling of the CD1 epitope does not give insight in the antigen presenting function of LC.

By in situ hybridisation HIV infected mononuclear cells expressing viral RNA were detected in six of seven lymph nodes and in seven of 14 blood samples of AIDS and ARC patients (Harper et al. 1986). These results demonstrated the presence of only 0,01% to 0,001% positive cells of the cell populations (Harper et al. 1986). It appears, however, that the number of latently infected cells containing the viral genome is at least ten times higher (Kunze et al. 1986).

We showed the presence of labelled cells in the connective tissue of the oral mucosa in two of 26 HIV infected patients. Due to the labelling of cells with monoclonal antibodies against HIV core-(p24) transmembrane-(gp41) and envelope protein (gp120) it is suggested that these mononuclear cells expressed HIV. Although double labelling studies were not performed, it seems likely that these cells were T-lymphocytes in the first patient (Fig. 4), whereas the stained cells in the second patient (Fig. 5) could be a LC due to the typical dendrite. The presence of HIV expressing cells in the connective tissue of the oral mucosa could be explained by the fact that latently infected cells invaded the oral mucosa together with uninfected cells of the immune system. It has been shown that latently HIV infected lymphocytes can be stimulated like normal lymphocytes and that stimulation is necessary for production of detectable amounts of virus (Zagury et al. 1986). This finding was underlined by the results of Kunze et al. (1986) who detected only one p24-positive cell in unstimulated peripheral blood lymphocytes of HIV infected patients only in one out of five patients, while after mitogen stimulation all HIV infected patients revealed 4 to 28 p24-positive cells/1000 peripheral blood lymphocytes. It might be assumed that oral candidiasis or viral infections, (HPV, EBV, CMV) lead to a continuous antigenic stimulation of the local immune system in the oral mucosa. This stimulation may activate latently HIV infected mononuclear cells causing expression of HIV as it was seen in



two out of 26 patients. Furthermore it was demonstrated by Braathen et al. (1987) in tissue culture that HIV infected LC produce immunohistochemically detectable amounts of virus structural proteins after mitogen stimulation. Compared with the large number of HIV positive lymph nodes observed in ARC and AIDS patients by Harper et al. (1986) we could only demonstrate the presence of HIV in a small number of biopsies. This is obviously due to the low number of immunocompetent cells in oral mucosa compared with lymph nodes. The finding of one positive control reaction was unexpected and underlines the necessity to perform control reactions on every biopsy and every antibody. As yet HIV expressing cells were detected in peripheral blood, lymph nodes, brain and LC of the skin. In the present study we were able to demonstrate the expression of HIV in the oral mucosa. Further studies are necessary to explain the probable significance of the presence of HIV in skin and oral mucosa, because this organ is the largest in man and might represent a reservoir for HIV.

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**United States Patent** [19]

Connelly et al.

[11] **Patent Number:** 5,597,688[45] **Date of Patent:** Jan. 28, 1997[54] **CELL FIXATIVE AND METHOD OF ANALYZING VIRALLY INFECTED CELLS**[75] **Inventors:** Mark C. Connelly, Doylestown, Pa.;  
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Houston G. Brooks, Jr., Somerset,  
both of N.J.[73] **Assignee:** Ortho Diagnostic Systems Inc.,  
Raritan, N.J.[21] **Appl. No.:** 467,799[22] **Filed:** Jun. 6, 1995**Related U.S. Application Data**[62] **Division of Ser. No.** 859,212, Mar. 27, 1992, Pat. No. 5,422,277.[51] **Int. Cl.<sup>6</sup>** ..... C12Q 1/70; G01N 33/53;  
G01N 33/569[52] **U.S. Cl.** ..... 435/5; 435/7.1; 435/7.24;  
435/975; 530/388.2[58] **Field of Search** ..... 435/5, 7.1, 7.2,  
435/7.24; 436/501, 800, 807; 530/387.1,  
388.1, 388.3, 388.7, 389.1, 389.6, 388.2[56] **References Cited****U.S. PATENT DOCUMENTS**

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*Primary Examiner*—Mary E. Mosher*Assistant Examiner*—Henry E. Auer[57] **ABSTRACT**

The present invention provides a novel cellular fixative composition and method of cellular fixing and cellular analysis wherein cells may be fixed for further analysis without destroying the cell's surface markers, cellular morphology, and the cell's light scattering properties. Treatment of cells with the fixative as described herein also allows antibodies or other desired components to enter the cell through the cellular membrane, without allowing important contents of the cell to escape. As an added benefit, the fixative composition of the invention may also kill or at least inactivate or reduce the activity of deadly virus contained in the cell. In its broadest aspect, the presently claimed fixative composition comprises at least three components, and in more preferred embodiments, four components. In a particularly preferred embodiment, the fixative composition described herein comprises at least one compound suitable for increasing the permeability of a cellular membrane, at least one compound that facilitates transportation of components across cellular membranes, at least one detergent, and at least one compound having the structure:

**9 Claims, 6 Drawing Sheets**

FIG. 1

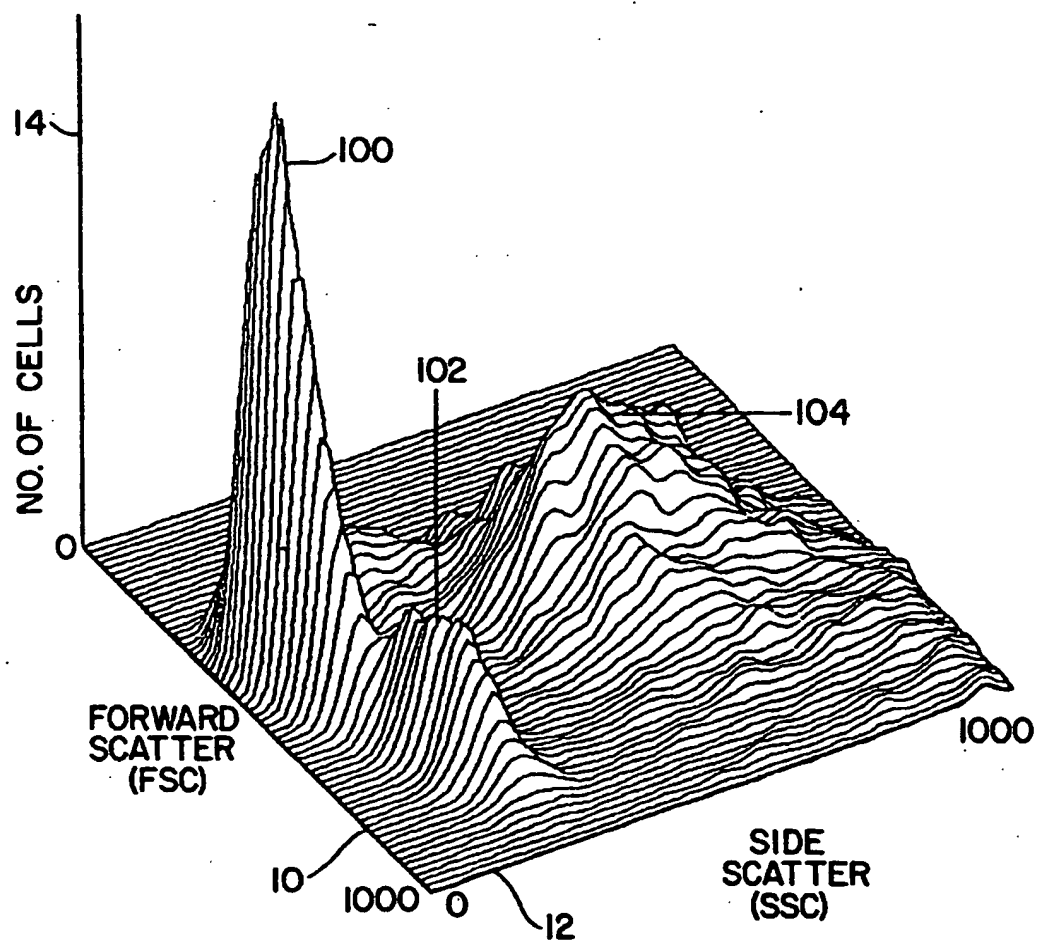


FIG. 2

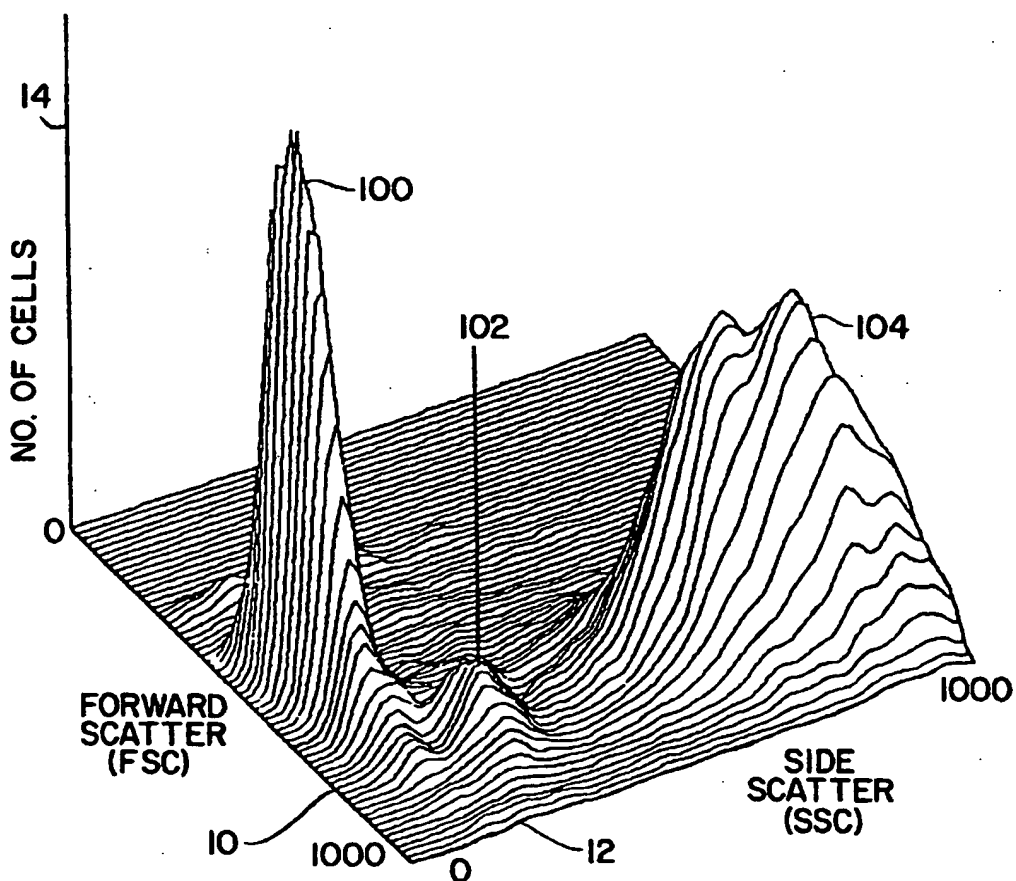


FIG. 3

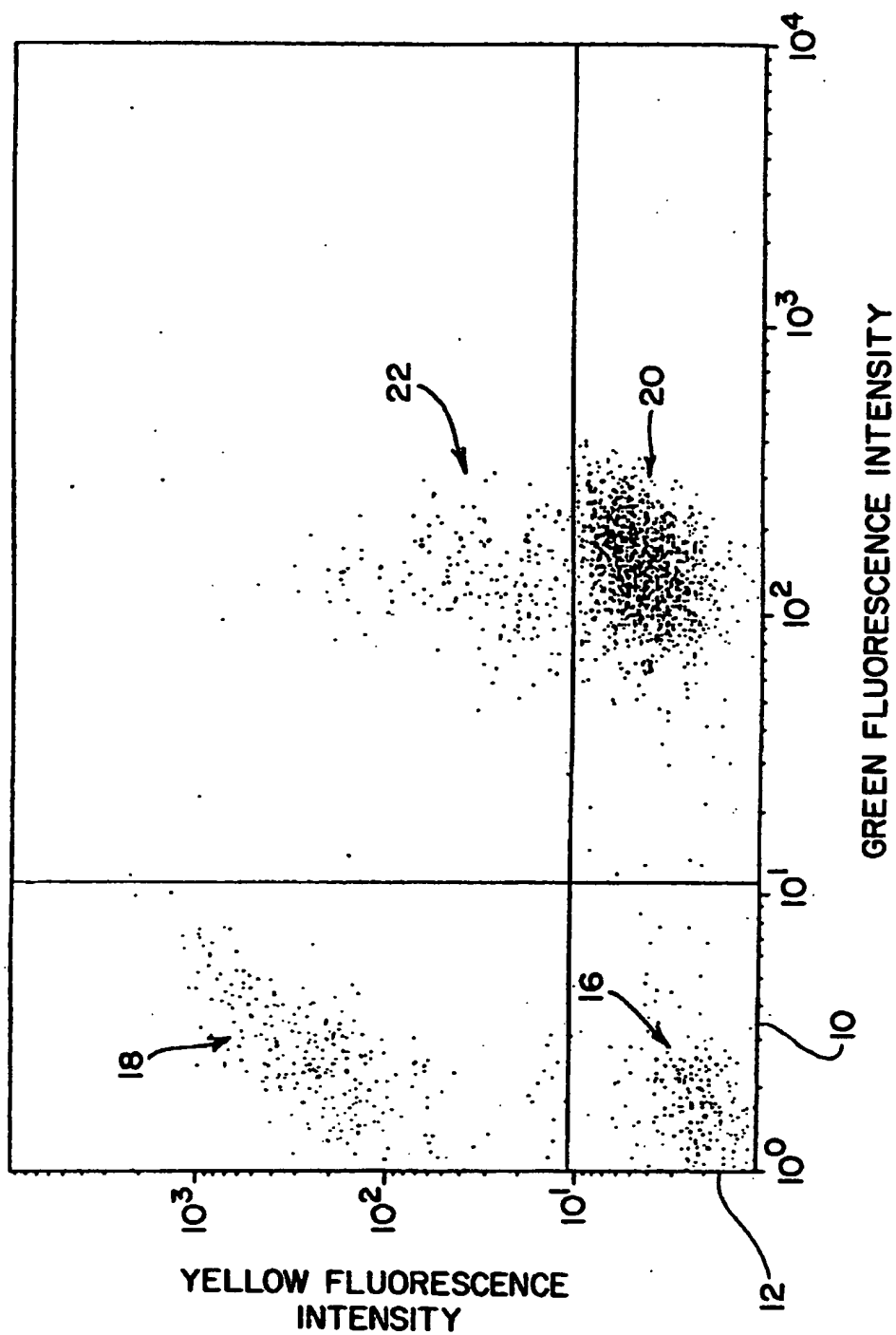


FIG. 4b

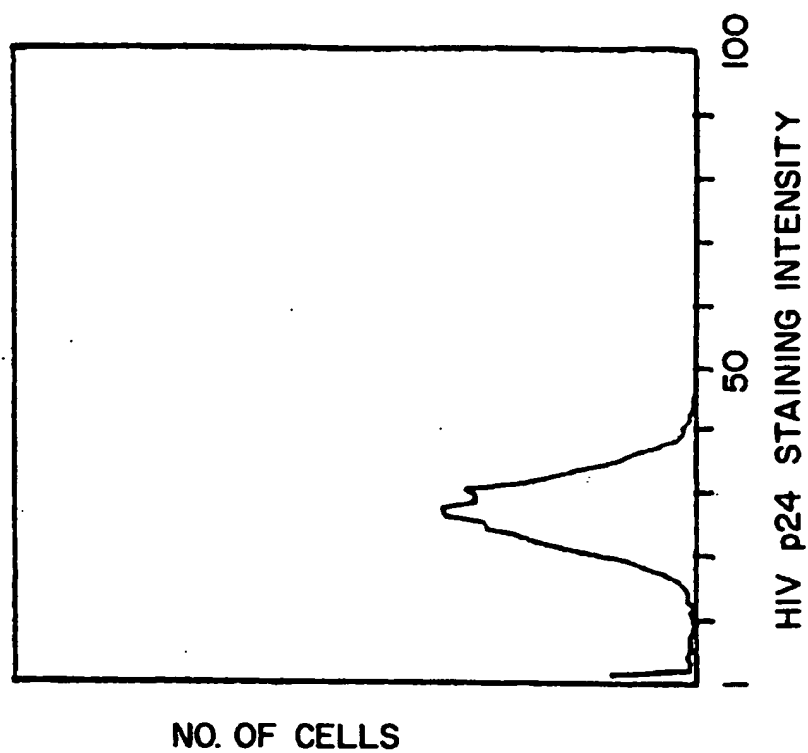


FIG. 4a

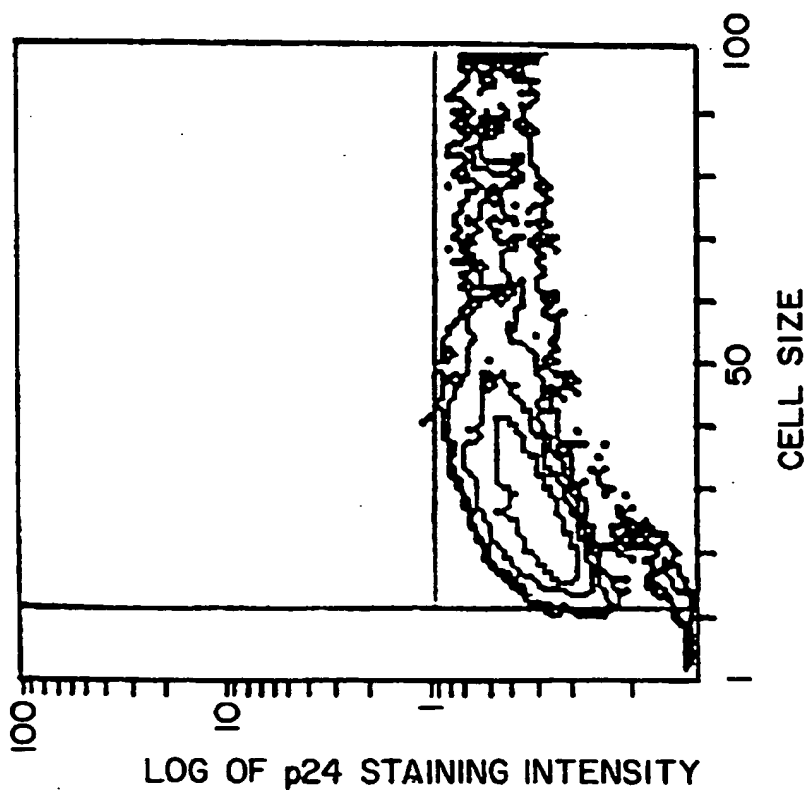


FIG. 5b

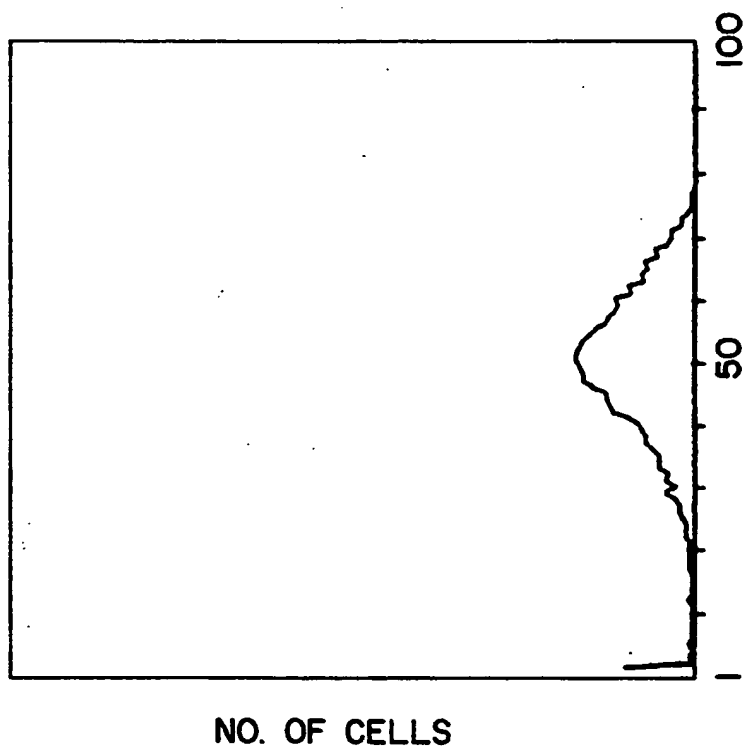
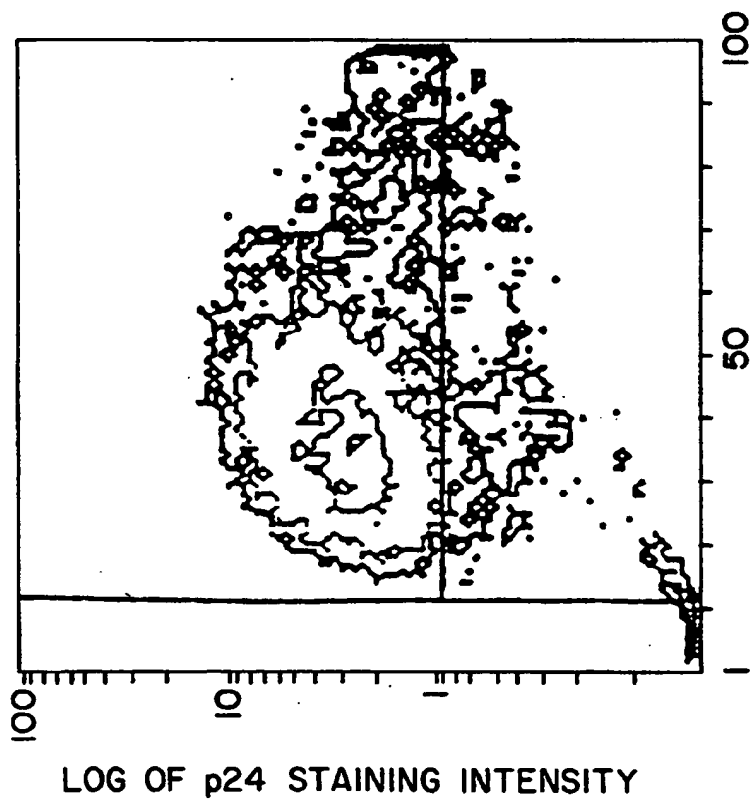
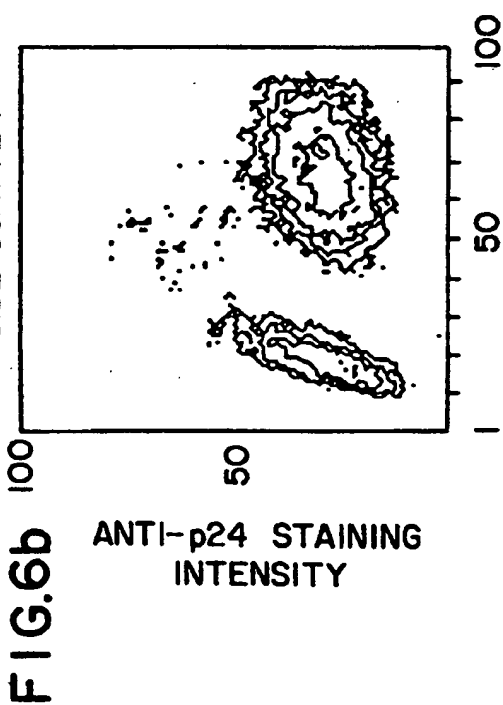
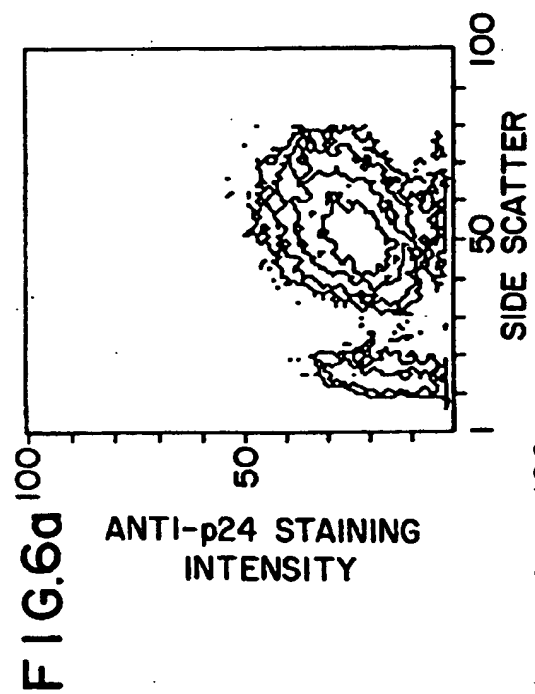
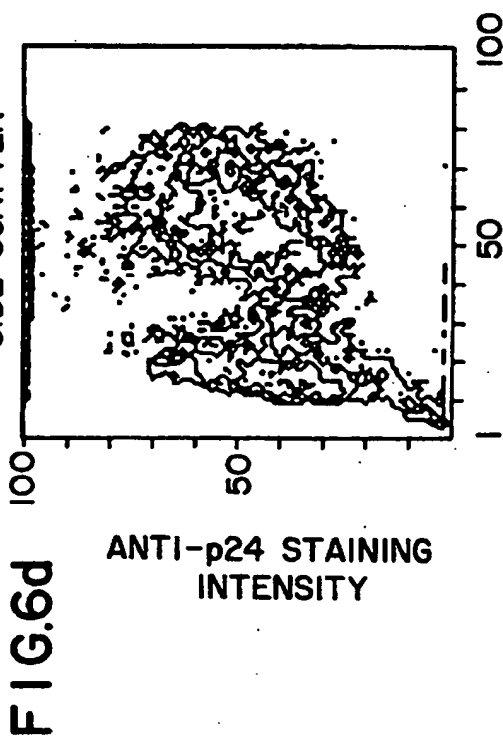
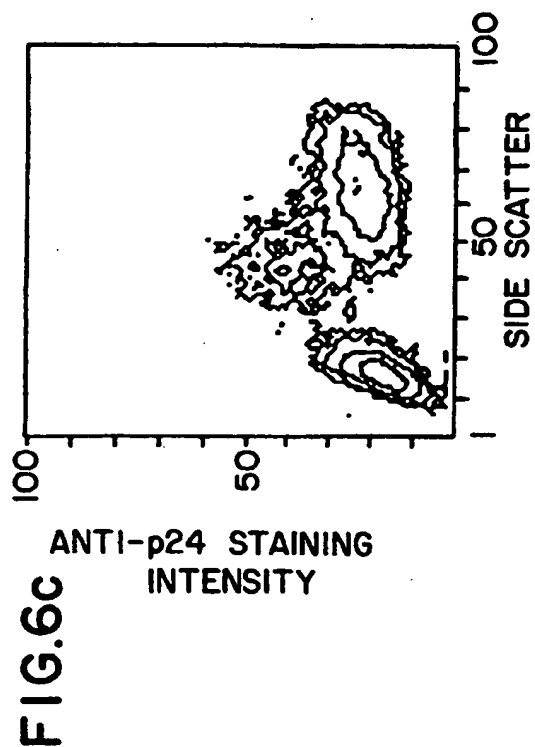


FIG. 5a







## CELL FIXATIVE AND METHOD OF ANALYZING VIRALLY INFECTED CELLS

This is a division of application Ser. No. 07/859,212, filed Mar. 27, 1992, now U.S. Pat. No. 5,422,277 which is hereby incorporated by reference.

The present invention relates to a cell fixative that allows the user to fix the cells, without substantially destroying cellular properties such as the cell's surface markers, morphology, and light scattering properties. In certain preferred embodiments, use of such a fixative composition would allow antibodies or other components of interest to enter into the cell, by rendering the cellular membrane permeable to these relatively large components, without releasing the cellular contents. The fixative and methods of cellular analysis described herein are particularly useful in the staining of cells to determine whether or not they are infected with a virus, the extent of such infection, and whether or not a patient is responding to treatment for a virus infection by the monitoring of that patient's blood cells for certain parameters of viral infection.

### BACKGROUND OF THE INVENTION

Since the outbreak of AIDS and other viral-related disease states there has been an increased need for new and better methods of studying the etiology and pathology of viruses, as well as therapeutic monitoring of treatment regimes for arresting or modifying in some way the effect these viruses have on cells, and ultimately the patient. For example, it is often desirable to assay the percentage of cells infected. A high virus burden usually means the disease is rampant, while a low virus burden might mean that the particular disease is in its early stages, or is responding to therapeutic treatment, and so on. Conventional assays for measuring virus burden have to date been merely extrapolative in nature. For example, cells may be cultured and titrated out for analysis. In these assays, it is common to employ polymerase chain reaction amplification techniques, in an attempt to quantitate copies of DNA (as the provirus form) or RNA present, the precept being, the more RNA, the more virus. However, results obtained with techniques of this sort can only indicate without distinction, a small quantity of infected cells having a high quantity of virus burden, or a larger quantity of cells with a low virus burden per cell. Another drawback is that a determination in accordance with this technique does not provide information as to whether or not the virus infection is replicative or abortive, since one does not know "how many" or "which" cells are infected. Hence, true virus burden assessment, as well as virus activity cannot be had with this technique. Furthermore, it is difficult, cumbersome, and costly to implement, all without giving results that are acceptable in sensitivity.

Virus infection may also be monitored by monitoring the quantity of a viral component such as p24 (in the case of HIV-1) present in a patient's serum. However, this technique is grossly extrapolative and often given to false negatives. For example, a patient may demonstrate a short spike in p24 concentration at the beginning of infection, when the virus is replicating, but before that patient's antibody response to this virus. Within a period of 5 days to about 2-3 weeks, the patient will start to make antibodies to p24. These antibodies bind to the p24 in plasma and either remove it from circulation or block its detection in immunoassays. Accordingly, there is a very short window in which to detect the p24 antigen component, as the patient will test negative once he is making antibodies to the p24. It is not until the patient

becomes so compromised that he can no longer produce antibodies to the p24 component, that the test begins to again indicate a positive result for the p24 antigen. Unfortunately, the patient prognosis is very grim at this point, as the disease has progressed past the stage of responding to any therapeutic treatment.

Hence, there is a continuing need for better tools and techniques for measuring viral burden in cells, and the viability and replicability of the virus in those cells. This information is invaluable in determining the progress of the viral infection and its response to treatment. Further, there is a definite need to monitor these parameters at a very early stage in the disease progression, and to continue this monitoring unimpeded throughout the path of infection.

In particular, there is a specific need for routine monitoring of virus load in HIV-infected individuals, preferably, through the use of a fixative that inactivates the virus and thus, increases the safety of handling samples containing this deadly virus. This information will be used by physicians to categorize HIV disease states, monitor and document progression, assess prognosis, and possibly to better tailor effective therapeutic regimens on an individual basis. Additionally, pharmaceutical companies and researchers require just such an assay for use in clinical trials, to determine rapidly if a proposed therapeutic agent is both safe and effective at controlling virus load.

By way of background in cell analysis techniques useful in monitoring a patient, it is noted that conventional flow cytometry is a technique quite suitable for such a task. The fundamental concept of flow cytometry is that cells or subcellular components in aqueous suspension are made to flow at high speed through a sensing region where optical or electrical signals indicative of important biologic properties are generated. These signals are analyzed and accumulated for evaluation. The cells may be fluorescently stained, although other dye systems may be employed, (see U.S. Pat. No. 4,933,293) and no staining is necessary for light-scatter measurements or electrical sizing. Generally, hydrodynamic methods are used to force the cells to move in almost identical trajectories at uniform speeds through a focal spot of intense illumination capable of exciting fluorescent emission from the fluorochrome used. A laser is the typical light source. The cell receives uniform illumination for a very short period of time and emits a burst of fluorescence and scattered light of this duration over all angles. A fraction of the light emission per cell is captured by an optical arrangement and one or more photosensors generating electrical signals proportional to the optical signals. Since the fluorescent light emission occurs at longer wavelength than the incident light while the scattered light experiences no wavelength change, these two signals can be separated with filters and measured independently and simultaneously for each cell. The electrical pulses are shaped, amplified, measured, and either displayed or stored for later analysis. Flow sorting can also be accomplished, to sort different cellular populations from a sample. Typically, the cell suspension is forced out of a tiny orifice and forms a high-speed liquid jet in air. Optical sensing is usually done in the jet in air close to the orifice outlet, and is basically identical to the method used in the flow cytometer just described. Applied ultrasonic vibration causes breakup of the jet into uniform droplets, which traverse a region of high (and constant) electric field intensity. Decision-making and charging circuits electrically charge only droplets containing selected cells; droplets containing unwanted cells and empty droplets remain uncharged. The electric field deflects the charged droplets, which contain the desired cellular subpopulation, into one

container while all the other droplets go to another container. In this way specific subpopulations of high purity can be obtained for further biologic study, such as morphologic or biochemical analysis (The above paragraph is taken from: *Flow cytometry and Sorting*, Melamed et al. editors, John Wiley and Sons, 1979, pp.11-14).

The above-described technique may be used with a patient's cells, to analyze for the presence of viral antigens. Interfering antibodies present in such patient's blood sample are simply washed away with the serum prior to this flow cytometric analysis. However, virion particles, if present, are in the cellular cytoplasm, and sometimes the nucleus. In order to look at the viral antigens inside the cell, the cellular membrane must be permeated to allow antibodies against the virus to enter the cell. Using the prior art techniques of the past, all or a portion of the cellular surface would be stripped away to allow the large antibody to enter. Typically this is done through the use of agents such as methanol or other alcohols which tend to extract lipids and precipitate proteins. Such agents basically turn the cell into a bead of protein, and in this manner provide access to the proteins that may be present. However, in so doing, the cell's surface characteristics are destroyed.

The present invention provides a cellular fixative and fixing technique which fixes cells without substantially destroying that cell's cellular surface characteristics, all the while allowing large molecules, such as antibodies, to enter the cell. This is accomplished without the concomitant release of the virion particles from the inner cell. Thus, a single treatment reagent is provided herein, which is capable of permeating the cell and fixing it, while preserving both the immunoreactivity and light scatter of such cell. The fixative and method of cellular analysis described herein is particularly well-suited for use in flow cytometry analysis.

### SUMMARY OF THE INVENTION

The present invention provides a novel cellular fixative composition and method of cellular analysis comprising at least one:

#### I. compound of the General Structure:



Wherein:

$X=SO_3H, SO_2NH_2, SO_2NHR, SO_2NR_2, SO_2OR, SO_2OAr, SO_2NHC_6H_5, SO_2N(C_6H_5)_2, COOH, COOR, COOC_6H_5, COOAr, CN, OH, OR, OCOR, OCONH_2, OCONHR, OCONR_2, OCONHAr, OCONAr_2$

$R=H$  or alkyl chain containing 1-6 carbon atoms;

$Ar=$ one to three aromatic rings, fused or non-fused and either the same or different, and comprising benzene rings, heterocyclic rings or rings containing one or more heteroatoms either the same or different, which is (are) nitrogen (N), oxygen (O) or sulphur (S), said rings substituted with  $R_1, R_2, R_3, R_4$ , and  $R_5$ ;

$R_1, R_2, R_3, R_4, R_5=$ are either the same or different, and are  $NO_2, COR, COOR, COOH, CONH_2, CONHR, CONR_2, CHO, X, OH, OR, Ar, R$ , or  $CF_3$ .

In preferred embodiments, the fixative composition further comprises at least one:

II. alcohol-free cellular fixative;

III. at least one compound suitable for increasing the permeability of a cellular membrane; and/or

IV. at least one compound that facilitates transport of components across cellular membranes.

In certain most preferred embodiments, a fixative composition comprising one or more components selected from each of the four above-mentioned categories is provided, especially when the alcohol-free cellular fixative component is high-grade formaldehyde, and the compound to facilitate transport across the cellular membrane component is dimethylsulfoxide. Also provided are methods of cellular analysis wherein a cell sample is fixed with the fixative composition as described herein, and the cells so fixed are then interrogated with labeled binding ligands to detect the presence or absence of various cell surface markers and/or intracellular components, and especially when that cellular analysis is accomplished through the use of flow cytometry. Results obtained using the aforementioned cell analysis may be applied to disease diagnosis and monitoring.

### BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a three-dimensional plot obtained with the use of a FACScan flow cytometer demonstrating the distribution of white blood cell clusters in a live, unfixed cell population.

FIG. 2 is a three-dimensional plot obtained with the use of a FACScan flow cytometer demonstrating the distribution of white blood cell clusters in a cell population fixed with the fixative composition of the invention.

FIG. 3 is a cytogram obtained with the use of a FACScan, demonstrating the results of a double antibody staining assay.

FIG. 4a is a contour plot, obtained with a FACScan, showing results of staining of uninfected cells reacted with anti-p24 monoclonal antibody.

FIG. 4b is a histogram of the same data as depicted in 4a, plotting the number of cells versus the relative fluorescence intensity.

FIG. 5a is a contour plot, obtained with a FACScan, showing the results of staining of persistently HIV-infected cells reacted with anti-p24 monoclonal antibody.

FIG. 5b is a histogram of the same data as presented in 5a, plotting the number of HIV-infected cells versus their relative fluorescence intensity.

FIGS. 6a, 6b, 6c, and 6d are contour plots, obtaining on a FACScan, showing the results of staining of blood from three HIV-infected patients (6b, 6c, and 6d), in three various stages of disease infection, and the results obtained with a sample from a control, noninfected hospital patient (6a).

### DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a novel cellular fixative composition, method of cellular fixing and cellular analysis, method of viral burden monitoring and monitoring of disease progression in a patient, as well as reagents useful therefore. Cells may be fixed for further analysis without substantially destroying the cell's cellular properties such as surface markers, cellular morphology, and the cell's light scattering properties. Treatment of cells with the fixative as described herein also allows antibodies or other desired components to enter the cell through the cellular membrane, without allowing a substantial amount of the contents of the cell to escape. Cells fixed with the composition of the invention, although dead, frequently demonstrate properties during cellular analysis that are very similar to those demonstrated by "live cells". For example, the differentiation of cell types based on their light scatter properties is still intact. Further, the morphology is very similar to that of live cells,

with much less swelling and shrinkage than is typically displayed by fixed cells. In sum, one can often analyze the cells fixed in accordance with the present invention in much the same manner as one would analyze a live cell population.

In its broadest aspect, the presently claimed fixative composition comprises at least one component that will serve to fix a cell's cytoplasm quickly, so as to maintain the integrity of the cellular contents in their normal position, without major disruption. This is almost analogous to "freezing" the cytoplasm in place. One or more compounds suitable for this purpose may be selected from the following classes of aromatic compounds:

A compound of the General Structure:



Wherein:

$X=SO_3H, SO_2NH_2, SO_2NHR, SO_2NR_2, SO_2OR, SO_2OAr, SO_2NHC_6H_5, SO_2N(C_6H_5)_2, COOH, COOR, COOC_6H_5, COOAr, CN, OH, OR, OCOR, OCONH_2, OCONHR, OCONR_2, OCONHAr, OCONAr_2;$

$R=H$  or Alkyl chain containing 1-6 carbon atoms

$Ar=$  one to three aromatic rings, fused or non-fused, either the same or different, and comprising benzene rings, heterocyclic rings or rings containing one or more heteroatoms either the same different, which is (are) N, O, or S, said rings substituted with  $R_1, R_2, R_3, R_4,$  and  $R_5$ ; and  $R_1, R_2, R_3, R_4, R_5=$  are either the same or different, and are  $NO_2, COR, COOR, COOH, CONH_2, CONHR, CONR_2, CHO, X, OH, OR, Ar, R$  or  $CF_3$ .

The fixative composition may comprise one or a combination of compounds selected from those having this General Structure.

In more preferred embodiments the fixative composition may comprise one or a combination of compounds selected from those having the General Structure and wherein:

$R_1, R_2, R_3=$  one, two or three groups, each either the same or different, and is (are)  $-NO_2, COR, COOR, COOH, CONH_2, CONHR, CONR_2, CHO, SO_3H, SO_2NH_2, SO_2NHR, SO_2NHAr, OH, OR, CF_3,$  or  $R$ .

In certain other preferred embodiments,  $R_1, R_2, R_3=$  one, two, or three groups, each either the same or different, and is (are)  $NO_2, COR, COOR, COOH, CONH_2, CHO, SO_3H, SO_2NHR, SO_2NR_2, SO_2NHAr, OH, OR,$  or  $R$ , with the proviso that  $R_3$  is not  $NO_2$  when  $R_1$  and  $R_2$  are  $NO_2$ . Explosive and other undesirable properties are inherent in tri-substituted  $NO_2$  compounds, and it is therefore preferable to avoid this substitution in certain instances, unless the undesirable properties can be neutralized in some way.

In particularly preferred embodiments,  $X$  is  $SO_3H, COOH,$  or  $OH$ , and  $R_1, R_2, R_3$  are the same or different and are  $NO_2, COOR, COOH, CONH_2, CONHR, CONR_2, CHO, OH, R,$  or  $X$ , with the proviso that when  $R_1$  and  $R_2$  are  $NO_2$ , then  $R_3$  is other than  $NO_2$ .

Particularly preferred compounds belonging to this generic class are 2,4-Dinitrobenzene sulfonamides, Dinitrophenols, 3,5-Dinitrosalicylic acid, 2,4-Dinitrobenzoic acid, 5-Sulfosalicylic acid, 2,5-Dihydroxy-1,4-benzene disulfonic acid, 3,5-Dinitrobenzoic acid, 8-Hydroxyquinoline-5-sulfonic acid, 4-Nitrophenol, 3,5-Dinitrosalicylaldehyde, 3,5-Dinitroaniline, Paratoluene sulfonic acid, 2-Mesitylene sulfonic acid, 2-(Trifluoromethyl) benzoic acid, 3,5-Dinitrobenzonitrile, and 2,4-Dinitrobenzene sulfonic acid. More particularly preferred are Dinitrobenzaldehyde, Dinitrobenzene sulfonic acids, Dinitrobenzoic acids, and specifically 3,5-Dinitrobenzoic acid, 2,4-Dinitrobenzoic acid,

2,4-Dinitrobenzene sulfonic acid, 2,6-Dinitrobenzene sulfonic acid, 3,5-Dinitrobenzene sulfonic acid, and 2,4-Dinitrophenol.

Preferred embodiments of the fixative composition of the invention comprise one or any combination of said aforementioned compounds, dissolved or otherwise dispersed in a vehicle that is compatible with the compound and cells and is suitable to make a homogeneous liquid composition containing such one or more compounds.

A substantial number of species belonging to this generic class of compounds may be obtained commercially from such chemical suppliers as Aldrich Chemicals (Milwaukee, Wis.), Fluka (Switzerland), Janssen (New Jersey and Belgium), Eastman-Kodak (Rochester, N.Y.), Lancaster Chemicals (Windham, N.H.), and other such fine chemical suppliers. One skilled in the art of organic synthesis will also understand that these compounds may be chemically synthesized *de novo*, or in part if intermediates to this component are first obtained commercially from suppliers as mentioned above. Suitable techniques for chemical syntheses of these types are described in such treatises as "Synthetic Organic Chemistry", by Wagner and Zook, John Wiley & Sons, Inc., New York, 1953; "Chemistry of Carbon Compounds", by Rodd, Elsevier Publishing Co., Amsterdam, 1954 (vol. III, part A); "The Organic Chemistry of Sulfur" by Chester M. Suter, John Wiley & Sons, Inc., New York, 1944; "Organic Syntheses", John Wiley & Sons, Inc. New York, to name but a few. One skilled in the art will understand that in many cases, the chemical name of a compound one desires to synthesize may be looked up in the table of contents or index of such treatises or lab manuals, for the correct page in the volume detailing that compound's synthesis. In most cases, the original papers describing the various syntheses are included in the bibliography sections of such works, and may also be consulted if desired.

In more preferred embodiments, the fixative composition of the invention comprises a second component. The second component of the fixative composition of the invention is one, or a combination of alcohol-free compounds, each of which is generally described in the art as a "fixative", and which acts by attaching to proteins and cementing their structure. The cementing mechanism of action accorded to these alcohol-free compounds may vary, and includes reaction of free amines, reaction with lipids, or cross-linking of the protein. Of these fixatives may be mentioned singly or in any combination formaldehyde, paraformaldehyde, glutaraldehyde, acrolein, glyoxal, malonaldehyde, diacetyl, polyaldehydes, carbodiimides, diisocyanates, diazonium compounds, diimido esters, diethylpyrocarbonate, maleimides, benzoquinone, metallic ions and other complexes such as chromium, mercury, osmium tetroxide, palladium chloride, uranium, and the like. Preferred as the component for inclusion in the fixative composition of the present invention are one or more amine reactive aldehydes such as glutaraldehyde, acrolein, formaldehyde, paraformaldehyde, and the like. Especially preferred is a high grade alcohol-free formaldehyde. One skilled in the art will understand that the above-mentioned components are commercially available through such vendors, *inter alia*, as Sigma Chemicals in St. Louis, Mo., Polysciences of Warrington, Pa., Aldrich Chemical of Milwaukee, Wis., and the like. A useful treatise for the chemistry of these and other commonly known fixatives may be found in *The Chemistry and Practice of Fixation*, from Histochemistry Theoretical and Applied, A. G. Everson Pearse, Vol. 1, 4th Edition, Publishers Churchill Livingstone, Edinburgh, London & New York, 1980, pp. 97-158. Concentrations of the subject components are those

concentrations generally used in the fixation of tissue, and concentrations preferably a little less than generally used, so as to afford a more "gentle" fixation. For example, in the case of the reactive aldehydes, preferred concentrations in percent as measured by weight per volume are generally about 0.1% (w/v) to about 4% (w/v). More preferred concentrations are about 0.2% (w/v) to about 2% (w/v) and particularly preferred concentrations range from about 0.5% (w/v) to about 1.6% (w/v).

In most preferred embodiments of the fixative composition of the invention, a third or even fourth component is added. The third component of the fixative composition of the present invention is a compound or combination of compounds selected from one of two groups of compounds. The first group may be functionally described as those compounds that facilitate transport of components across cellular membranes. The second group may be described as a detergent or surfactant, such as a non-ionic detergent. In the most particularly preferred embodiments, the fixative composition comprises at least one component from each of these two categories, totaling four separate components in all.

Of the compounds that facilitate transport of components across the cell membrane, may be mentioned those that decrease the surface potential of lipid monolayers. Of these, may be mentioned water-soluble or water-insoluble "fusogenic" compounds, such as dimethylsulfoxide, sulfolane, 1-methyl-2-pyrrolidinone, polyethylene glycol (PEG), ethyleneglycol, and the like. This component generally renders the cellular membrane more permeable to low molecular weight compounds, and facilitates entry of such compounds into the cell's cytoplasm, at the same time it prevents the cell from swelling. The previously described components, namely the fixative component and the component of General Formula I, can then enter rapidly and fix the contents of the cell before they could spill out in any substantial measure. Preferred among the compounds that facilitate transport across the cell membrane is dimethylsulfoxide, because it is less fusogenic than some compounds that promote transport across a membrane, has less of an effect on light scatter, is stable, and does not react to any great extent with the other components of the fixative. Suitable concentrations are those concentrations that will not cause membrane fusion in substantial measure. Preferred concentrations range from about 1% (v/v) to about 20% (v/v), with about 5% (v/v) to about 15% (v/v) especially preferred.

Suitable detergents to serve as either the third or fourth component of the fixative composition are one or a combination of detergents that will render the cellular membrane permeable to large molecules of about 200 kD to about 1000 kD, and especially large molecules generally described as binding ligands useful in binding to and thus detecting cell surface markers or intracellular components, and the like. Good examples of such binding ligands are labeled antibodies, labeled DNA and RNA probes, specific substrates, co-factors, and the like. Typical labels for such binding ligands are fluorescent compounds such as the phycobiliproteins (including phycoerythrin), and fluorescein isothiocyanate (FITC), radioactive labels, enzymatic, biotin-avidin labels, and the like, all well known to the art. Accordingly, the detergent or combination of detergents should be capable of permeating the cell surface to the degree necessary to accommodate entry of a molecule of this size. However, this component should accomplish permeation at a concentration that will enable it to act in concert with the other components of the fixative composition, so as to avoid, in substantial measure, extraction of lipids or other components from the

cell's interior. If too much of the cellular components of a cell's interior is extracted, the light-scattering properties of the cell will be detrimentally affected.

Preferred for use herein as this third or fourth component, are one or a combination of zwitterionic or non-ionic surfactants such as sodium cholate, deoxycholates, CHAPS, saponin, and polymers of ethylene oxide, such as ethoxylated alcohols, ethoxylated alkyl phenols, ethoxylated amines and amides, polyoxyethylene sorbitans of the "Tween" series, such as monolaurate (Tween 20), monopalmitate (Tween 40), monooleate (Tween 80), and polyoxyethylene 23 lauryl ether (Brij™ 35), polyoxyethylene ether W-1 (Polyox), and the like. One skilled in the art will understand that a good description of compounds belonging to the foregoing classifications, and vendors from whom such compounds may be commercially obtained may be found in "Chemical Classification, Emulsifiers and Detergents", McCutcheon's, *Emulsifiers and Detergents*, 1986, North American and International Editions, McCutcheon Division, MC Publishing Co., Glen Rock, N.J., U.S.A. and Judith Neugebauer, *A Guide to the Properties and Uses of Detergents in Biology and Biochemistry*, Calbiochem®, Hoechst Celanese Corp., 1987. Preferred among these are the polyoxyethylene sorbitans of the "Tween" series available from Sigma Chemicals, or the Triton™ series, available from Rohm and Haas of Philadelphia, Pa., and especially Triton™ X-705, Triton™ X-100, and Triton™ X-114, available commercially from many vendors. Preferred concentrations of these range from about 0.001 to about 0.2% (w/v), with about 0.05 to about 0.1% (w/v) particularly preferred.

The fixative composition of the invention is prepared by selecting one or more components as described herein, and then physically dispersing the desired one or more components into a suitable buffer or other liquid vehicle, such as a isotonic medium. Examples of such suitable vehicles include phosphate buffered saline, saline, MOPS, HEPES, HEPES, Hank's Balanced Salt Solution, RPMI, and the like. Denaturation or deformation of the cells should be avoided and a concentration of the compound in the vehicle should be that which is suitable to fix cells in accordance with the techniques described herein. The components should be dispersed homogeneously or actually solubilized in the liquid vehicle. Amounts of the various components of the fixative composition, as chosen in accordance with the details above, are combined in a suitable vessel under mixing conditions. One skilled in the art may routinely choose the sequence in which each component is added to the mixture. For example, in the most particularly preferred embodiments, a phosphate buffered solution is first added to the vessel under conditions of continuous mixing followed by DMSO, DNBS (as a solid), Tween™, and formaldehyde. This sequence of addition of components to prepare the fixative composition has been found to be particularly suitable. The mixing process is typically carried out at room temperature, but one skilled in the art could routinely vary the temperature.

The fixative composition of the invention may be used to fix prokaryotic or eukaryotic live cells of any type, and especially bacteria and mammalian cells. The cells are contacted with a suitable amount of the fixative composition, and allowed to remain in such contact for at least about one minute, with about twenty minutes to about two hours of incubation preferable. Temperatures maintained during such incubation are generally 0° C. to about 37° C., with room temperature preferable. Amounts of fixative composition suitably used vary from (relative amounts in volume/

volume of cell sample to fixative composition) about 1:1 to about 1:100 (v/v), with about 1:10 to about 1:30 (v/v) particularly suitable. Excess cell fixative composition may be removed by any conventional means, including centrifugation, and the cells may be resuspended in a suitable cell suspension medium, such as buffer, if desired, prior to cell analysis. One skilled in the art of cellular fixation may routinely vary these aforementioned cell treatment parameters to obtain the desired cell fixation without substantial destruction of cellular properties. In preferred embodiments, the fixative composition of the invention is used in the fixing of bone marrow and blood cells, and especially white blood cells. The cells so fixed may then be examined by any suitable technique known to the art. Examples of such cell analysis would be through the use of a microscope, including an electron microscope, light microscopy, immunofluorescence, flow cytometry, and the like.

A preferred method of cellular analysis utilizing the fixative composition of the invention is through the use of flow cytometry. By way of background, flow cytometers exist in a variety of configurations depending on their intended use. However, they all contain four essential features. Each has a source of incident light, a fluid stream that carries the sample to the point where the incident light is focused, an optical system that converts light reflected off or emitted from the sample into electronic signals, and finally, a means to output the information to the user. Lasers are the most common source of light because they deliver light of a single wavelength at very high intensity. Although flow cytometers have been used on a wide variety of samples, including bacterial cells, chromosomes, tumor cells and others for illustrative purposes, this discussion will emphasize the analysis of a sample comprising a single cell suspension of white blood cells.

In general, to analyze a suspension of cells, the sample is introduced into the center of a fluid stream through a narrow injection port. The stream serves two purposes; first to bring the cells to the point where both the incident light and optical system are focused, and second to orient the cells in single file. Because cells travel in single file, they are interrogated by the light beam one at a time, and the light reflected or emitted by each cell is measured and recorded independent of the other cells in the sample. As a cell is carried into the laser light, it breaks up the beam and scatters it in all directions. Although the cell scatters the light, it does not change the light's wavelength. Therefore, scattered light travels along a different path but maintains the same wavelength as the incident light beam. Typically, the cell suspension has been treated with either fluorescent dyes or antibodies coupled to fluorescent dyes. These fluorescent compounds absorb light of one wavelength and emit light of a longer wavelength. Thus, if the cell contains the fluorescent dye or has reacted with the antibody-dye complex, in addition to scattering light, the cell will emit light of the wavelength characteristic of the fluorescent dye. The amount of light emitted is proportional to the quantity of fluorescent molecules present. The quantity of light emitted by each cell is measured and referred to as its "fluorescence intensity", which is depicted as a numerical value on a scale that is designated by the user, such as 1 to 10,000.

Flow cytometers typically contain several photomultiplier tubes to convert photons into electric impulses. Filters placed in front of each photomultiplier select the wavelength of light to which the photomultiplier will respond. The incident beam is prevented from entering the detection system containing the photomultiplier tubes; therefore, only light scattered by a cell, or emitted from it as fluorescence,

is allowed to reach the photomultipliers. Scattered light is collected in two places; at a low angle only slightly deflected from the axis of the incident beam and at a right angle to the beam. The former is called low angle or forward scatter; whereas, the latter is called right angle or side scatter. The reason for measuring scattered light at two different angles is because forward and side scatter provide different information about the cell. How much light is scattered in the forward direction depends on the cells' size and its index of refraction. The bigger the cell the more light it scatters in the forward direction. Light scattered at right angles to the beam is less dependent on cell size than on the complexity and number of intracellular organelles.

Lymphocytes are small cells and therefore have low forward scatter. Lymphocytes also exhibit low side scatter because they have very little cytoplasm and regularly shaped nuclei. Monocytes are larger, their cytoplasm grainier, and their nuclei more complex in shape than lymphocytes. As one would predict, monocytes scatter more light than lymphocytes in both the forward and side directions. Granulocytes may vary in size from being as small as lymphocytes to being larger than monocytes. The broad range of forward scatter exhibited by granulocytes reflects this size heterogeneity. However, because of their cytoplasmic granules and irregular shaped nuclei, granulocytes exhibit the most side scatter of the three main cell types in blood. The light scatter of each cell as it passes through the beam is measured and may be plotted on a graph of forward scatter (FSC) versus side scatter (SSC). Such a plot allows the operator to separate and identify cell types and direct the instrument to display and save data from one, two, or any combination of cell types.

The use of light scatter is illustrated in FIGS. 1 and 2, which are three-dimensional representations of the number of cells having light scattering properties as measured on a FACScan flow cytometer available from Becton Dickinson Corporation. Forward light scatter was plotted on the abscissa (X-axis) 10, versus SSC on the ordinate (Y-axis) 12. The number of cells is represented on the Z-axis, 14. The intensity of FSC and SSC for each cell was measured and given a relative value on a scale of 1 to 1000. FIG. 1 shows the results using live unfixed cells, with 100 representing the lymphocyte cluster, 102 representing the monocyte cluster, and 104 representing the granulocyte cluster respectively. FIG. 2 shows the results using cells after having been fixed with the fixative composition of the present invention. Although fixed and made permeable, it was evident that cell clusters remained well defined. Because light scatter depends on factors such as cell size, cytoplasmic complexity and the cell's index of refraction, it was not surprising to find that the light scatter of fixed cells had varied a little from unfixed cells. However, it was surprising to find that after fixation, not only could the three populations of cells be resolved by light scatter alone, but that the separation of lymphocytes, monocytes and granulocytes was improved.

Information that a cell is a lymphocyte, monocyte or granulocyte is useful, but more information than that may be required for a complete analysis. There are many different kinds of lymphocytes and they have quite distinct functions. Some lymphocytes produce antibodies (B-cells), while others serve to regulate the immune system or carry out certain effector functions (T-cells). To be able to identify and enumerate functional subsets of lymphocytes is essential in immunologic research and in diagnosing and monitoring diseases of the immune system. Yet, the functional subsets of lymphocytes cannot be differentiated solely on their light scatter. Fortunately, lymphocytes that differ in function also

differ in the antigens they express. As a result, antibodies have been developed that react with specific functional subsets of T-cells. Helper T-cells, for example, are critical to the overall function of the immune system. As their name implies, their role is to help the immune system mount an effective response against foreign substances such as viruses, bacteria and parasites. Helper T-cells express the CD4 molecule on their surface and anti-CD4 antibodies react with these helper T-cells. The flow cytometer can exploit this fact to determine how many of an individual's lymphocytes are of the helper type. A fluorescent molecule is coupled to the anti-CD4 antibody. A commonly used molecule is fluorescein isothiocyanate (FITC). This compound will absorb blue light and emit green light. A sample of white blood cells is reacted with anti-CD4-FITC and then analyzed in the flow cytometer. Cells parading in single file are illuminated by blue laser light and the amount of blue light scattered in the forward and side directions determined. Simultaneously, the amount of green light emitted by the cell is measured. The CD4 positive lymphocytes that reacted with the antibody emit green light in addition to the blue light they scatter. Non-helper T-cells scatter light in a manner identical to helper T-cells, but because no antibody is present they do not emit green light. The flow cytometer can be made to identify a cell as a lymphocyte by virtue of its light scatter profile, and then count the number of lymphocytes that emit green light versus the number that do not. In this way, the proportion of lymphocytes that are helper cells may be determined.

Not every cell that reacts with anti-CD4-FITC is a helper T-cell. Monocytes also express CD4 on their surface, but at a lower concentration than helper T-cells. The lower concentration of CD4 means less antibody binds to monocyte surfaces and so they emit less green light. This makes them dimmer than helper T-cells, but brighter than CD4 negative cells. However, because monocytes have greater forward and side scatter than lymphocytes, the user can direct the flow cytometer to accept or ignore data from cells having the desired light scatter profile. The ability to discriminate light scatter profiles allows the user to get an accurate count of CD4 positive lymphocytes without interference from CD4 positive monocytes. This may be particularly important in some diseases such as Acquired Immunodeficiency Syndrome (AIDS). Helper T-cells may appear less fluorescent or dimmer in AIDS patients than in healthy people. Were it not for the light scatter profile identifying a cell as a lymphocyte, dim CD4 helper T-cells might be otherwise mistaken for monocytes, which could lead to an underestimate of the number of helper T-cells.

A more complicated assay would employ a second antibody added simultaneously. In this case, the second antibody would have a different specificity from the first, and be coupled with a dye that emits light of a different color. Typically, phycoerythrin (PE) is used for this purpose, because it too absorbs blue light, but fluoresces yellow. In this way, one can determine whether a cell reacts with either the FITC labeled antibody, the PE labeled antibody or both. For example, the antigen known as DR is not expressed on T-cells unless the cells have been activated. In contrast, the DR antigen is expressed on most B-cells. If white blood cells are reacted with anti-CD3-FITC and anti-DR-PE antibodies, all four possible combinations of reactivity would be expected. Some lymphocytes would not react with either antibody and would only scatter light. The non-activated T-cells would react with anti-CD3 antibody and emit only green light. B-cells would react with anti-DR antibody and emit only yellow light. However, activated T-cells would

react with both antibodies and emit both green and yellow light. Such a situation is illustrated in FIG. 3, which is a cytogram obtained with the use of the FACScan. Here white cells have been reacted with anti-CD3-FITC and anti-DR-PE and the fluorescence of the lymphocytes, selected as lymphocytes on the basis of their light scatter, has been plotted with each cell's green fluorescence intensity on the abscissa 10, and its yellow fluorescence intensity on the ordinate 12. Unreactive cells cluster at the origin 16. B-cells are displaced vertically, in a cluster 18 distinct from unreactive cells but directly above it. Unactivated T-cells are displaced along the abscissa in a cluster 20 distinct from unreactive cells but directly along side of them. Finally, activated T-cells 22 emit just as much green light as unactivated T-cells because they express the same amount of CD3, but they are also displaced vertically because they co-express the DR antigen, and having reacted with the anti-DR-PE antibody, emit yellow light.

The situation may be made even more complex by the inclusion of a third antibody or a DNA/RNA stain that absorbs blue light and emits red light. This arrangement of reagents is not discussed in detail because it does not illustrate any principles of flow cytometry not already covered by the previous discussion of one and two color reagent analysis; however, it does serve to illustrate how complex and sophisticated flow cytometric analysis of cells may become. By way of example, if the third reagent is a DNA stain, then the amount of DNA in a cell may be measured. The amount of DNA is dependent upon whether the cell is at rest, synthesizing DNA in preparation for cell division, or is about to divide. Quantitating the amount of DNA allows the user to selectively examine cells in various stages of the cell cycle. It then becomes possible to determine whether certain antigens are always expressed by a cell, or only present during restricted portions of the cell cycle. Such data is analyzed essentially as done for one and two color assays, but with greater appreciation for the complexity of simultaneous reactions being measured.

In accordance with the cellular analysis method of the present invention, a cell sample is first obtained from any of a variety of cell sources. Such sample may or may not be purified or otherwise pre-treated in accordance with conventional cell analysis protocols. In the preferred embodiment, a blood sample is obtained. Example II below describes a typical blood sample collection procedure. It should be emphasized, however, that the current method may be carried out without ficolling red blood cells or lysing red blood cells from the whole blood sample prior to fixation (see Example IV). The cellular fixative composition of the invention is mixed with the cells using a quantity of fixative sufficient to fix the cells without substantially destroying their surface membranes.

By way of illustration only, a sample of a patient's whole blood may be obtained by routine venipuncture, drawing the blood sample into a tube containing an anticoagulant such as EDTA or heparin. Approximately 0.1 mls of the whole blood is then mixed with 2 mls of fixative solution prepared in accordance with the teachings herein, and maintained at room temperature. The blood/fixative mixture is incubated for 30 minutes at room temperature, centrifuged to pellet the cells, and the supernatant fluid then removed by aspiration. The cells so fixed are resuspended in wash buffer and allowed to sit for about 10 minutes. The cells are then washed two times in phosphate buffered saline and serum. Optionally, any remaining red blood cells may be lysed using standard lysing procedures.

This basic procedure may be varied in many ways. For example, it may be desirable to first dilute the whole blood sample with an isotonic diluent before proceeding with the fixation method steps. Lysed whole blood may also serve as the sample. The whole blood would be first treated to lyse red blood cells by standard methods and procedures such as by ammonium chloride treatment. The unlysed white blood cells are then washed and resuspended in an isotonic medium, in a concentration that is preferably  $1-2 \times 10^7$  cells/ml, and then treated in accordance with the above procedure. Whole blood samples may also first be treated by standard methods on Ficol-Hypaque, to remove red blood cells and granulocytes. Lymphocytes and monocytes are then suspended at preferably about  $1 \times 10^5$  to about  $1 \times 10^8$  cells/ml, and more preferably about  $1-2 \times 10^7$  cells/ml, and fixed as above. One skilled in the art of blood cell analysis will understand and appreciate these and other conventional techniques for blood sample preparation.

Cell analysis is then conducted in accordance with the previously described techniques and concepts and the following examples, just as though the cells were unfixed, live cells.

Using the fixative composition of the invention, and the techniques of flow cytometry, clinicians may study the viral load infecting a sample of a patient's cell population, for example, white blood cells. In certain preferred embodiments, a study of HIV-infected individuals may be performed using the methods described herein. For example, A. Venet et al. in "Quantitation of Cellular Viral Load: Correlation With CD4 Cell Count", from *Viral Quantitation in HIV Infection*, Ed. J. -M. Andrieu, John Libbey Eurotext, Paris 1991, pp 27-36, describes the usefulness of monitoring viral load. However, he employs the cumbersome, hazardous, and costly techniques of cell culture to attempt to elucidate the required information from various patients' blood cell analyses.

Using techniques of flow cytometry with the resulting data analysis as depicted in FIGS. 1, 2, and 3, and the fixative composition as described herein, a clinician may analyze a patient's blood sample to determine cell phenotypes, which cells are infected with virus, and how many cells are growing the virus. One could even determine "how much" virus is actually in certain cells, in some cases. Using this information, the clinician may then make decisions of diagnosis, therapeutic monitoring, patient prognosis, and the like. For example, two patients having a similar CD4 positive count may demonstrate vastly different clinical presentations and progression. Using the techniques and compositions of the invention, it is possible to determine each patient's respective virus burden. For example, one patient may have a CD4 count of 200, but may demonstrate less than 0.1% CD4 positive cells that are actually replicating virus. The second patient may have 4% of their CD4 positive cells replicating virus. In this latter case, the disease progression may be further along, and the prognosis poorer. Or, the virus may have acquired a drug resistance, and the clinician could decide to change the treatment regimen.

Many viruses may be monitored in a patient's sample for their own sake or simultaneously with the HIV virus or some other virus of interest, in a similar manner. Interestingly, it may be the existence of other viruses, such as hepatitis, that are first detected in an HIV patient who is asymptomatic. Sub-clinical viremia due to one or more other viruses may also indicate that a patient's immune system is breaking down. This may occur before the HIV virus itself becomes rampant. Also, HIV in the presence of an increasing viral burden from other viruses could be a sign of true immuno-

suppression. Thus, the techniques and compositions as discussed herein provide a rapid overall assessment of the immune system's competence or function.

A test kit containing the fixative composition of the invention in conjunction with suitable cell markers is also provided for the convenience of the researcher and clinician. By way of example, a useful combination reagent kit comprises a vessel containing the fixative composition of the invention, along with vessels containing Orthomune™ OKT4, Orthomune™ OKT8, and Orthomune™ OKDR, which are antibodies available from Ortho Diagnostic Systems Inc., Raritan, N.J. This kit is then utilized in accordance with the techniques herein described to monitor various types of virus infection. The user will understand that this kit is used in conjunction with one or more antibodies to all or a part of the virus of interest. Using such a combination reagent kit, a patient's immune system may be panoramically monitored. In preferred combination kit embodiments, the kit itself may include antibodies or panels of antibodies to various viruses of interest. For example, in the detection of HIV, one or more antibodies to the HIV virus, such as anti-p24, anti-p17, anti-gp41, anti-gp120, and the like are also included in the kit in conjunction with certain antibodies to cellular surface markers such as the aforementioned Orthomune™ OKT antibodies.

The following are more specific embodiments of the present invention but should not be considered limitative thereof.

#### EXAMPLE 1

##### Comparison Examples Showing Failure of Current Art to Preserve Light Scatter Properties and Staining of Cell Surface Antigens

##### Comparative Example 1: Cell Population Discrimination After Paraformaldehyde Fixing

Four grams of paraformaldehyde (PF) were placed in 400 ml of phosphate buffered saline (PBS). The 1% (w/v) PF suspension was heated, with constant stirring, until all PF was in solution.

Blood was collected from a single donor into heparinized tubes then centrifuged ( $457 \times g$ ,  $22^\circ \text{C}$ , 10 minutes). The buffy coat from two blood collection tubes was pooled in a 50 mL centrifuge tube and the total volume adjusted to 35 mLs using Hanks Balanced Salt Solution (Mediatech), supplemented with 5% (v/v) horse serum (HBSP). Ten mLs of ficoll-hypaque (Lympho-paque, Nocomed Pharma As) was layered underneath the buffy coat being careful to prevent mixing of the Ficoll-buffy coat interface. The tube was spun at  $1170 \times g$  for 20 min at  $22^\circ \text{C}$ . Peripheral blood lymphocytes and monocytes present at the ficoll interface were collected. The cell pellet containing red blood cells and polymorphonuclear leukocytes was discarded. Lymphocytes and monocytes were diluted in ice cold HBSP then pelleted by centrifugation ( $457 \times g$ ,  $4^\circ \text{C}$ , 8 minute). Cells were again washed in HBSP.

Cells were washed once in PBS, then resuspended in 4 mLs of PBS ( $4 \times 10^7$  cells/mL). The cells were separated into 4 1 mL aliquots. The first tube received 1 mL of PBS and tubes 2 through 4 received 1 mL of 1% paraformaldehyde. All tubes were incubated for 30 min at room temperature. All tubes were washed twice in PBS. Tubes 1 and 2 were put in ice until needed. Cells in tubes 3 and 4 were resuspended in 1 mL of PBS. To tube 3 was added 1 mL of 0.5% (w/v) Nonidet P40 (NP40; BDH Limited), and it was incubated 30



minutes at room temperature. To tube 4 was added 6.6 mLs of methanol ( $-70^{\circ}\text{C}$ .) and it was incubated 30 minutes at  $0^{\circ}\text{C}$ .

All cells, regardless of treatment, were washed twice in 50 mLs of HBSP plus 2% (v/v) human AB serum (457 $\times$ g,  $4^{\circ}\text{C}$ ., 8 minutes). All cells were resuspended at a concentration of  $1 \times 10^7$  cells/mL in HBSP plus 2% human AB serum (HBSP-AB). 200  $\mu$ L of each cell suspension was incubated with 20  $\mu$ L of the fluorescein isothiocyanate (FITC) conjugated monoclonal antibodies: Control, OKT3, OKT4, and OKT11 (Ortho Diagnostic Systems Inc., Raritan, N.J.). Cell suspensions were incubated for 30 minutes at  $0^{\circ}\text{C}$ ., then washed twice by centrifugation (457 $\times$ g,  $4^{\circ}\text{C}$ ., 8 minutes) in ice cold HBSP-AB. Each suspension was resuspended in 0.5 mL of HBSP-AB.

The light scatter properties and level of fluorescent antibody binding was determined through routine flow cytometric techniques using a FACScan (Beeton Dickinson) flow cytometer. The FACScan was equipped with an argon laser emitting light at 488 nm. Fluorescence detector 1 (FL1) equipped with a 530/30 nm band pass filter was used for quantitating FITC fluorescence. Unless otherwise indicated light scatter measurements were made on a linear scale of from 0-256 units and fluorescence was measured on a logarithmic scale of 1 to  $10^4$  units.

Forward light scatter (FSC) and the level of FITC fluorescence for unfixed live cells treated with a Control-FITC antibody was measured. Cells pre-treated with 1% paraformaldehyde (PF) were also analyzed in the same manner.

Although the cells had been treated with 1% PF, two distinct cell populations were discriminated by FSC. Lymphocytes were seen with a FSC around 105 (on a scale of 1 to 256) whereas monocytes had a FSC around 180. Cells treated with 1% PF displayed more nonspecific binding of control-FITC antibody than did live, unfixed cells.

Experiments were also conducted wherein cells were treated with OKT3 antibody and measured in a similar manner. OKT3 reacts with the molecule CD3 on the surface of T cells. CD3 is not present on B cells or monocytes, so these cells fail to react with OKT3.

Lymphocytes that bind OKT3 were evident in the PF treated cells. The average fluorescence of PF treated cells was lower than untreated cells; and the positive and negative populations were not as well separated following PF treatment. The lower binding intensity of OKT3 suggests CD3 may have been damaged by PF treatment, but not altered to the point where OKT3 will no longer bind. Additional evidence that lymphocytes and monocytes were distinguished on the basis of their FSC was the finding that no CD3 positive T cells were present in the monocyte cell cluster (FSC approx. 180 on a scale of 1-256).

Cells were also treated with the antibody OKT4. OKT4 binds to CD4 on the surface of the helper subset of T cells. OKT4 bound to the PF treated lymphocytes. Unlike CD3, CD4 is expressed at a low level on the surface of monocytes. Thus, live, unfixed monocytes showed dim fluorescence with OKT4. PF treated monocytes did not appear to bind OKT4. However, because CD4 is expressed at low levels on monocytes, even a small amount of damage to CD4 would render these cells negative. As was the case with CD3, CD4 does appear to have been damaged by PF treatment, but not destroyed.

OKT11 staining was also performed and measurements taken in the same manner as before. OKT11 binds CD2 on the surface of T cells. On live unfixed cells, the OKT11

positive population displayed only modest fluorescence. No OKT11 staining was observed on PF treated cells. Treatment of cells with PF appears to have altered CD2 in a way that prevents OKT11 from binding to its epitope.

#### Comparative Experiment 2: Methanol Permeation

In order to visualize intracellular antigens, it is not enough to simply fix cells. Cells must also be made permeable to molecules as large as antibodies. The methods most frequently employed to permeabilize cells are methanol or detergent treatment of fixed cells (reviewed in Jacobberger, J. W., (1989) Cell cycle expression of nuclear proteins. In A. Yen (ed.), Flow cytometry: advanced research and clinical applications. CRC Press, Inc. Boca Raton Fla.). Methanol further fixes proteins, randomizes their conformation and extracts membrane lipids. Detergent treatment extracts membrane lipids, creating holes where antibodies may pass freely in and out of the cell.

Immunostaining and light scatter properties of cells fixed in 1% PF versus cells fixed in 1% PF followed by methanol permeabilization was measured. The ability to discriminate lymphocytes from monocytes on the basis of their FSC is diminished by methanol treatment. In addition, methanol treated cells had increased nonspecific binding of control-FITC antibody. The most important finding, however, was that methanol treated cells no longer reacted with OKT3, OKT4, and still did not react with OKT11.

#### Comparative Example 3: Detergent Treatment

Immunostaining and light scatter properties of cells fixed in 1% PF versus cells fixed 1% PF followed by detergent treatment (0.5% NP40) was also measured for comparison purposes. The ability to discriminate lymphocytes from monocytes was diminished by detergent treatment. Detergent treated cells also showed a marked increase in nonspecific control-FITC binding over cells treated with 1% PF. OKT3 did bind detergent treated cells, however, there was very little separation between the positive and negative populations. Poor FSC separation of lymphocytes and monocytes made it unreliable to separate these populations by light scatter. As a result, the percent OKT3 positive lymphocytes could not be determined accurately following detergent treatment. Even though OKT3 still bound CD3 positive lymphocytes, it could not be known with certainty how many of the CD3 negative cells were truly lymphocytes and how many were monocytes with FSC altered by detergent treatment. The effect of detergent treatment on monocyte light scatter properties became evident when detergent treated cells were treated with OKT4. OKT4 bound lymphocytes, and to a lesser extent monocytes, as expected since both contain CD4 positive cells. Three populations of cells, and their OKT4 binding, were measured successfully. The three populations that were determined were CD4 negative lymphocytes with no OKT4 binding, monocytes having a low density of CD4 with intermediate staining, and CD4 positive lymphocytes with bright fluorescence. However, in the detergent treated cells, many monocytes had light scatter values between 80 and 140 FSC; the same range of FSC values obtained for lymphocytes. It was clear from this result that following detergent treatment, the lymphocyte light scatter cluster was heavily contaminated with monocytes.

#### EXAMPLE 2

##### Preservation of Cellular Antigenic Specificities Following Fixation With the Composition of the Invention

The fixative reagent was prepared by adding constituents to distilled  $\text{H}_2\text{O}$  to final concentrations: 14% (v/v) dimethyl

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sulfoxide (DMSO, Sigma Chemical Co.); 0.14% (w/v) polyoxyethylene sorbitan monolaurate (Tween 20, Aldrich Chemical Company); 39.2 mM 2,4-dinitrobenzene sulfonic acid sodium salt (DNBS, Aldrich Chemical Co.); 1.51% formaldehyde (Ultrapur 10% EM grade, Polysciences Inc.); 1.470 mM  $\text{KH}_2\text{PO}_4$ ; 2.683 mM KCl; 8.058 mM  $\text{Na}_2\text{HPO}_4$  and 67 mM NaCl. The pH of the fixative solution was adjusted to 7.4; and the solution was stored in an amber bottle overnight at room temperature.

Blood was collected by venipuncture directly into a tube containing K3-EDTA as the anticoagulant. The blood and anticoagulant was mixed and kept at room temperature until needed (approximately one hour).

Ten mLs of whole blood were placed in a 50 mL centrifuge tube. Whole blood was diluted with 40 mLs of freshly prepared buffered ammonium chloride lysing reagent (Ortho Diagnostic Systems Inc.). Blood was incubated 20 minutes at room temperature with occasional mixing. White blood cells were pelleted by centrifugation (457×g, 21° C., 8 minutes). White blood cells were washed twice by centrifugation (457×g, 21° C., 8 minutes) in phosphate buffered saline (PBS) at room temperature. Washed cells were resuspended in PBS at a concentration of  $2 \times 10^7$  cells/mL. The pool of white cells was separated into aliquots. Cells that were not to be fixed were diluted with an equal volume of PBS+5% (v/v) horse serum (PBS/S) and placed on ice until needed. The cells to be fixed were diluted with an equal volume of fixative reagent. Cells were mixed and incubated for 30 minutes at room temperature. After 30 minutes, fixed cells were washed twice in 50 mLs of PBS. Live and fixed cells were washed once in "Block Solution." Block solution was composed of 25% (v/v) goat serum+5% (v/v) horse serum +5% (w/v) bovine serum albumin (BSA). Live cells were resuspended to  $2 \times 10^7$  cells/mL in block solution and placed on ice for one hour. Fixed cells were also resuspended in block solution at  $2 \times 10^7$  cells/mL, but kept at room temperature for one hour.

#### DIRECT IMMUNOSTAIN

Cell surface antigenic determinates were stained using antibodies to cell surface markers, conjugated directly to FITC (OK-control, OKT3, OKT4, OKT11 and OKT3/OKDR-PE combination, all available from Ortho Diagnostic Systems Inc., Raritan, N.J.). 100  $\mu\text{L}$  of either live or fixed cell suspension was placed in a reaction tube and 10  $\mu\text{L}$  of the appropriate antibody or control added to the tube. Cells and antibody were incubated for 60 minutes. All incubations and washes using live cells were done at 0° C., whereas all incubations and washes using fixed cells were done at room temperature. After 60 minutes, cells were washed three times in PBS/S using 2 mL per wash. After the last wash, cells were resuspended in 0.5 mL of PBS/S and analyzed on a FACScan flow cytometer.

#### INDIRECT IMMUNOSTAIN

To determine whether fixation had made cells permeable to antibody while retaining their cytoplasmic antigens, live and fixed cells were reacted with mouse monoclonal antibodies specific for the cytoplasmic proteins gelsolin and vimentin. Anti-gelsolin (clone No. GS-2C4, Sigma Chemical Co.) and anti-vimentin (clone No. V9, Sigma Chemical Co.) were not conjugated to fluorescent dyes. Therefore, binding of these antibodies could not be determined "directly." Instead, binding of these antibodies to cells was determined "indirectly" by reacting treated cells with goat

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anti-mouse IgG-FITC. Immunostaining of cytoplasmic antigens was done by adding 10  $\mu\text{L}$  of Control IgG2a mouse antibody (10  $\mu\text{g}/\text{mL}$ ), or 10  $\mu\text{L}$  of anti-gelsolin (diluted 1/100 in block solution) or 10  $\mu\text{L}$  of anti-vimentin (diluted 1/60 in block solution) to 100  $\mu\text{L}$  of fixed or live cell suspension. Cells and antibody were incubated for one hour as described above, then washed three times in PBS/S using 2 mLs per wash. After the last wash, the supernatant fluid was removed by aspiration, and the cells resuspended in 100  $\mu\text{L}$  of block solution. Each suspension then received 200  $\mu\text{L}$  of goat anti-mouse IgG-FITC conjugate (F(ab')<sub>2</sub>, Sigma Chemical Co.) diluted 1/75 in block solution. Cells were again incubated for 60 minutes, then washed three times in PBS/S, using 2 mL per wash. After the last wash, cells were resuspended in 0.5 mL of PBS/S and analyzed on a FAC-Scan flow cytometer.

It is known in the art that fixed cells usually exhibit a substantial increase in either autofluorescence, nonspecific binding of antibody, or both. It is also known, the amount of nonspecific binding by antibody can be minimized with appropriate blocking reagents and cell washing conditions. In this example, all cells were treated with a blocking solution and washed in the same wash buffer.

In the interest of conserving space, time and avoiding undue repetition, only data from the lymphocyte cluster is discussed herein. The lymphocyte cluster was identified by FSC versus SSC and a "gate" or region was established to narrow the output of the computer analysis to those cells within the defined region or "lymphocyte gate." Although only the lymphocyte analysis is discussed, fixed lymphocytes were representative of how fixed monocytes and granulocytes react with anti-gelsolin, anti-vimentin, and antibodies against cell surface markers appropriate to their respective cell types.

OK-Control-FITC is a mouse IgG2a antibody that does not react with any known cellular antigens. Therefore, any green fluorescence emitted by cells following treatment with this antibody is due to nonspecific binding of antibody to cells. The nonspecific binding by live and fixed cells was determined. When reacted with OK-Control, 100% of live cells had a fluorescence intensity less than 3.92 (on a scale of 1 to  $10^4$ ), with a mean intensity for live cells of 1.20. Nonspecific binding of antibody to fixed cells was only slightly higher than that of live cells. 99.5% of fixed cells had fluorescence less than 13.82, with a mean fluorescence intensity of 3.99. The data therefore demonstrated that cells fixed and stained by the present reagent and methods do not bind significant quantities of antibodies nonspecifically.

Staining of the cell surface molecule CD3 by OKT3-FITC antibody was measured. 77.4% of live lymphocytes were positive for CD3, compared to 77.9% of the fixed lymphocytes. The mean fluorescence intensity for CD3 positive live cells was 188.83. The mean fluorescence intensity of CD3 positive fixed cells was 144.68; 23% lower than the intensity of live cells. Although the intensity of staining was modestly lower, the reagent and methods as described herein did not destroy the ability of OKT3 to bind CD3 on fixed cells; and enough OKT3 was bound by fixed cells that CD3 positive lymphocytes could be separated unequivocally from CD3 negative lymphocytes, based on their fluorescence intensity. This result is in marked contrast to cells fixed and made permeable by paraformaldehyde and methanol treatment as performed in the art, where the ability of OKT3 to bind CD3 had been destroyed completely (see Comparative Experiment 2 above).

Staining of the cell surface molecule CD4 by OKT4-FITC antibody was also performed. 55.1% of live lymphocytes

were positive for CD4, compared to 52.7% of the fixed lymphocytes. The mean fluorescence intensity for CD4 positive live cells was 69.57. The mean fluorescence intensity of CD4 positive fixed cells was 63.74; 8% lower than the intensity of live cells. Although the intensity of staining was modestly lower, the present invention's reagent and methods did not destroy the ability of OKT4 to bind CD4 on fixed cells; and enough OKT4 was bound by fixed cells that CD4 positive lymphocytes could be separated unequivocally from CD4 negative lymphocytes based on their fluorescence intensity. This result is in marked contrast to cells fixed and made permeable by paraformaldehyde and methanol treatment, where the ability of OKT4 to bind CD4 had been destroyed completely (see Comparative Experiment 2 above).

Staining of the cell surface molecule CD2 by OKT11-FITC antibody was also performed. 85.3% of live lymphocytes were positive for CD2, compared to 84.3% of the fixed lymphocytes. The mean fluorescence intensity for CD2 positive live cells was 49.95. The mean fluorescence intensity of CD2 positive fixed cells was 41.00; 18% lower than the intensity of live cells. Although the intensity of staining was modestly lower, the present invention's reagent and methods did not destroy the ability of OKT11 to bind CD2 on fixed cells; and enough OKT11 was bound by fixed cells that CD2 positive lymphocytes could be separated unequivocally from CD2 negative lymphocytes based on their fluorescence intensity. This result is in marked contrast to cells fixed and made permeable by paraformaldehyde and methanol treatment, where the ability of OKT11 to bind CD2 had been destroyed completely (see Comparative Experiment 2 above). In addition, when lymphocytes were treated with paraformaldehyde followed by NP40 solubilization of membranes, some CD3 and CD4 staining was observed, but no staining of CD2 by OKT11 was seen.

Cells were also incubated with two antibody specificities conjugated to fluorescent molecules having different emission spectra. Cells were incubated with OKT3-FITC and OKDR-PE simultaneously. Dot blot cytograms were obtained from the FACScan where staining of the cell surface molecule CD3 by OKT3-FITC antibody was displayed on the X-axis and staining of the cell surface molecule DR by OKDR-PE was displayed on the Y-axis. The dot plots, which were analogous to that shown in FIG. 3, were divided into 4 quadrants; upper left (UL), upper right (UR), lower left (LL) and lower right (LR). 78.7% (UR+LR) of live lymphocytes were positive for CD3, compared to 79.7% of the fixed lymphocytes. B-cells represented 6.8% of the lymphocyte population as determined by OKDR staining (upper left) of live cells. For fixed cells, B-cells represented 7.4% of the lymphocytes, in good agreement with the live cell population. There was also excellent agreement between the live and fixed cell populations on the percentage of activated T-cells, as determined by co-expression of the DR and CD3 antigens. 4.48% (upper right) of the live T-cells and 4.22% of the fixed T-cells were determined to have been activated. These results not only extend the list of cell surface antigens that may be detected post-fixation to include DR, but also serve to demonstrate that multiple antigenic specificities may be probed simultaneously when cells have been fixed using the reagents and methods as described herein.

Staining of intracellular antigens was done using an indirect immunostain procedure, as described above. Because of this, OK-Control-FITC was not the appropriate negative control. Instead, cells were incubated with an unconjugated control IgG2a antibody, washed and incubated

with the goat anti-mouse-IgG-FITC. 99.6% of live cells had a fluorescence of 3.92 or less, with a mean fluorescence of 1.21. 99.4% of fixed cells had a fluorescence of 12.86 or less, with a mean fluorescence of 5.84. As was seen with the direct antibody conjugate control, the data demonstrate that cells fixed and stained by the present reagent and methods do not bind significant quantities of antibodies nonspecifically.

Staining of the cytoplasmic molecule gelsolin by anti-gelsolin antibody demonstrated that 0.4% of live lymphocytes were positive for anti-gelsolin fluorescence, compared to 91.4% of the fixed lymphocytes. The mean fluorescence intensity of the anti-gelsolin negative live cells was 1.22. The mean fluorescence intensity of anti-gelsolin positive fixed cells was 50.45. Gelsolin is a cytoplasmic antigen not expressed on the cell surface. As a result, live cells didn't stain positive for gelsolin because antibody could not enter the cell. In contrast, the interior of the cell was accessible to antibody when cells were fixed by the reagent and methods of the present invention. Anti-gelsolin was a whole IgG molecule, not a fragment; therefore, molecules at least as large as 150,000 daltons could freely enter and leave these fixed cells.

Staining of the cytoplasmic molecule vimentin by anti-vimentin antibody demonstrated that 0.6% of live lymphocytes were positive for anti-vimentin fluorescence, compared to 92.0% of the fixed lymphocytes. The mean fluorescence intensity of the anti-vimentin negative live cells was 1.24. The mean fluorescence intensity of anti-vimentin positive fixed cells was 555.01. Vimentin is a cytoplasmic antigen not expressed on the cell surface. As a result, live cells didn't stain positive for vimentin because antibody could not enter the cell. In contrast, the interior of the cell was accessible to antibody when cells were fixed by the reagent and methods of the invention. It is important to note that vimentin has a molecular weight of 58,000 daltons, whereas anti-vimentin has a molecular weight of 150,000 daltons. Therefore, these results show that during fixation, small cytoplasmic proteins are retained by the cell and held in place, despite the cell having been made permeable to molecules the size of intact antibodies.

It has been seen that cells fixed by the reagent and methods of the present invention maintain sufficient light scatter properties to allow lymphocytes, monocytes and granulocytes to be discriminated, one from the other. Fixed cells allow free access of antibodies to internal cellular antigens, yet these same antigens have been fixed in place and are not washed out of the cells, even though the antigen may be smaller than an antibody molecule. Finally, cell surface molecules on fixed cells are intact and may be immunostained by one or more antibody molecules, providing for identification and quantification of white blood cell functional subtypes.

### EXAMPLE 3

#### Effect of Fixative Composition on Immunostaining of Cytoplasmic Antigens

#### Investigation Using Computer Aided Statistical Design and Analysis—"SEDA")

In order to examine the effect of the fixative composition on cytoplasmic antigen staining, a human cell line was used in place of white blood cells. A cell line was used because the expression of cytoplasmic antigens would be homogeneous. Therefore, differences in the percentage of cells scored positive, or the fluorescence intensity of individual

antigens, could be attributed to differences in the composition of the fixative rather than differences in antigen expression by heterogeneous cell types.

T-cells express CD3 on their cell surface. However, some T-cell tissue culture lines express cytoplasmic CD3 but little or no cell surface CD3 (Van Dongen et. al. Blood, 71: 603, 1988). The CEM T-cell line is one such cell line; having cytoplasmic but no cell surface CD3. CEM cells were grown, fixed with a variety of fixative formulations, then reacted with anti-CD3 (OKT3), anti-gelsolin and anti-vimentin.

The fixative reagent of the invention was prepared by adding constituents to distilled H<sub>2</sub>O to final concentrations as called for by the computer aided statistical experimental design (see Table E3-1). The fixative formulations were prepared the day before the experiment and stored overnight in the dark and at room temperature. Regardless of the fixative being tested, the method of fixation was as follows: CEM cells were pelleted (457×g, 21° C., 8 minutes), then washed twice by centrifugation (457×g, 21° C., 8 minutes) in PBS/S at room temperature. Washed cells were resuspended in PBS/S at a concentration of 2.5×10<sup>6</sup> cells/mL. The pool of cells was separated into 1 mL aliquots, then diluted with an equal volume of the appropriate fixative reagent. Cells were mixed and incubated for 30 minutes at room temperature. After 30 minutes, fixed cells were washed twice with 10 mLs of ice cold PBS. All cells were washed once more in PBS/S then resuspended to a concentration of 3.3×10<sup>6</sup> cells/mL.

#### Indirect Immunostain

Cells were reacted with OKT3 and the mouse monoclonal antibodies anti-gelsolin (clone No. GS-2C4, Sigma Chemical Co.) and anti-vimentin (clone No. V9, Sigma Chemical Co.). Immunostaining of cytoplasmic antigens was done by adding 5 uL of Control IgG2a mouse antibody (15 ug/mL), or 5 uL of OKT3, or 5 uL of anti-gelsolin (diluted 1/40 in PBS/S) or 5 uL of anti-vimentin (diluted 1/30 in PBS/S) to 100 uL of fixed or live cell suspension. Cells and antibody were incubated for one hour at 0° C., then washed twice in PBS/S using 2 mLs per wash. After the last wash, the supernatant fluid was removed by aspiration, and the cells resuspended in 250 uL of goat anti-mouse IgG-FTTC conjugate (F(ab')<sub>2</sub>, Sigma Chemical Co.) diluted 1/75 in PBS/S. Cells were again incubated on ice for 60 minutes, then washed three times in PBS/S, using 2 mL. per wash. After the last wash, cells were resuspended in 0.5 EL of PBS/S and analyzed on a FACScan flow cytometer.

#### Statistical Experimental Design and Analysis (SEDA)

Computer aided SEDA was used to further optimize the present invention. Using SEDA, more information could be obtained from a given set of experiments than could be obtained using more traditional methods.

The theoretical basis of SEDA was developed in 1960 by G. E. P. Box and D. W. Behnken. [Box, G. E. P., and D. W. Behnken (1960). Some new three level designs for the study of quantitative variables. *Technometrics* 2: 455-475.] Although main frame computers used to be required in order to make use of SEDA, it is now available commercially as a PC compatible software package. The software package used in these studies was "X-Stat Statistical Experimental Design/Data Analysis/Nonlinear Optimization"; available through Softpower Incorporated, John Wiley & Sons, Inc.

The essence of SEDA represents a departure from "traditional" experimental design. In a "traditional" experiment, the researcher first identifies all important mechanisms that may impact the result. Then a list is made of important variables to study and the performance parameters to be measured. For a problem as complex as cellular fixation, the list of variables and measured outcomes are long. Thus, a comprehensive optimization of the various parameters is better accomplished by the use of SEDA. For example, if three variables are to be studied, a traditional design would call for two variables to be held constant while the third is varied. If a linear relationship exists between the variables, and the variables are entirely independent, then to be able to predict performance, measurements need to be made at only two points for each variable. However, if the relationship between variables is not linear, the variables interact with each other or their relationship is not known, then each variable must be evaluated at a minimum of three points. Only if there are three points for each variable is it possible to determine if a linear or non-linear relationship exists. To test 3 variables at three levels requires 27 separate combinations. This approach is called a "full three level factorial design". The number of combinations in a full three level factorial design increases exponentially as the number of variables increase. With 4 variables, 81 combinations are required and 5 variables requires 243 combinations. As a result, researchers typically restrict the scope of a study to a manageable number of variables. Unfortunately, when only some of the variables are studied, the final result is determined fundamentally by whether the researcher chose wisely when deciding on the variables to study.

SEDA provides the researcher with the ability to study many more variables simultaneously by reducing the number of combinations required within the experiment. In the example of the experiment having 3 variables, the 3 variables may be called X, Y and Z. The range of values from low to high for X, Y and Z within the experiment may be thought of as defining the dimensions of a cube; with X representing the width, Y the height and Z the depth. A full three level factorial design would require a test at each corner of the cube and all midpoints (27 experiments in all). The Box-Behnken design requires testing only the combinations that represent the mid-point of each edge of the cube, and triplicate determinations of the combination that represents the center of the cube. This reduces the number of combinations from 27 to 15. The advantage of the Box-Behnken design becomes dramatic as the numbers of variables increase. A Box-Behnken design requires only 27 combinations for 4 variable and 46 for 5 variables; compared to 81 and 243 respectively for a three level factorial design. In addition, after the data has been collected and the computer selects mathematical models that correspond to the observed experimental results, SEDA can be used to predict the performance of the assay at any point on the surface or within the volume of the cube.

Experiments were designed using SEDA. A file was created where the variables to be studied (eg. formaldehyde, DNBS, DMSO and detergent concentrations) and the performance to be measured is., staining of cytoplasmic or surface antigen) were entered into the computer. After specifying the upper and lower limit for each variable, the software determined the concentration of each variable for each "run" within the experiment (see Table E3-1). The runs were then put in random order. After the laboratory portion of the experiment was completed, the measured performances corresponding to each run were entered into the computer. The computer determined those variables that had

the greatest effect on performance, those that had no effect, and those that interacted synergistically. Regression lines were fitted to the experimental data using linear, interactive and quadratic equations. The mathematical model giving the best fit to the data was chosen and carefully checked for how well it predicted the experimental results.

The computer model could be used to predict assay performance at any concentration or any combination of concentrations within the range of each variable tested. With this capability, countless combinations of reagent concentrations were tested by computer simulation. The ability of these simulated fixative formulations to allow staining of cytoplasmic antigens, or in some cases the degree of harm done to cell surface antigen staining, was predicted. Furthermore, minimum performance criteria were set and an algorithm in the software used to calculate the optimum combination of reagent concentrations to achieve any given desired performance. Once optimized, the computer was instructed to hold all variables at their optimal level except one. One variable was then varied over its entire range and its effect on performance plotted.

Staining of all three cytoplasmic antigens was observed at all concentrations of reagents tested; however, computer analysis of the observed experimental results predicted a preferred embodiment comprising 0.756% formaldehyde, 25.4 mM DNBS, 6.92% DMSO and 0.086% Tween 20 detergent. This computer predicted preferred embodiment was extremely similar to a preferred embodiment elicited through laboratory fixation experiments, wherein the concentrations of the relative components are 0.85% formaldehyde, 30 mM DNBS, 6.9% DMSO and 0.095% Tween 20 detergent. The latter fixative composition was tested on whole blood, whereas the computer modeling was based on the CEM cell line. The concentration of each reagent corresponding to the preferred embodiment was entered into the computer model. A computer generated plot of how formaldehyde concentration effects the fluorescence intensity of cytoplasmic CD3 staining by OKT3 demonstrated that fluorescence intensity declines rapidly as the formaldehyde concentration increases beyond 0.80%, even if all other constituents of the fixative remain at their optimal concentration. Other plots demonstrated the effect of the three other active ingredients in the fixative. It is important to note that the X and Y axes of each graph are different. The Y axis of the formaldehyde plot covers a mean fluorescence intensity range of 50 units, whereas the Y axis on the detergent plot covers a range of 400 units. Over the concentration ranges tested, detergent had the biggest effect on detection of cytoplasmic CD3; followed by DMSO, DNBS and formaldehyde. Detergent, DMSO and DNBS all served to improve CD3 detection in CEM cells. Formaldehyde had adverse effects on CD3 detection at concentrations greater than 0.80%.

Computer analysis of the factors influencing cytoplasmic staining of gelsolin was also performed. Gelsolin detection was insensitive to the concentration of formaldehyde tested in this experiment. A modest increase was predicted with increasing formaldehyde concentration, but the predicted increase was within experimental error. The same may be said of DNBS. Therefore, across the range of DNBS concentrations tested, DNBS was found to have little effect on detection of gelsolin in CEM cells. The concentration of DMSO had the biggest positive effects increasing the mean fluorescence an estimated 160 units. Detergent concentration increased fluorescence an estimated 110 units.

Computer analysis of the factors influencing cytoplasmic staining of vimentin was also performed. Vimentin detection

was very sensitive to formaldehyde concentration. A decrease in mean fluorescence intensity of an estimated 250 units was found. Concentrations of DNBS greater than 25 mM was estimated to increase mean fluorescence by as much as 200 units. DMSO also improved the detection of vimentin, increasing mean fluorescence an estimated 150 units. Increasing the DMSO concentration above 4.5% did not appear to help or hurt vimentin detection significantly in CEM cells. Detergent concentration had the biggest effect. An increase in mean fluorescence of 400 units was estimate as the detergent concentration was increased from 0.04% to approximately 0.08%. Higher concentrations were not predicted to improve vimentin detection.

The predictions of the computer model were verified for peripheral blood lymphocytes. White blood cells from a healthy donor were either washed in PBS/S or fixed at final reagent concentrations of 0.755% formaldehyde, 7% DMSO, 0.08% Tween 20 and either 0, 19.6 or 38 mM DNBS. All cells were fixed for 30 minutes at room temperature, then washed. All red blood cells from either fixed or live cell populations were removed by ammonium chloride lysis. The white blood cells were then immunostained using an indirect immunostain procedure and the antibodies anti-gelsolin and anti-vimentin, as described above.

The effect of fixation on the ability to detect cytoplasmic gelsolin and vimentin in peripheral blood lymphocytes is shown in Table E3-2. Less than 0.5% of live cells were stained with anti-gelsolin or anti-vimentin. However, 80 to 90% of fixed lymphocytes stained positive for these antigens. The concentration of DNBS during fixation did not effect the percentage of cells that bound either anti-gelsolin or anti-vimentin. Although the level of fluorescence for anti-gelsolin and anti-vimentin was much lower in this experiment than it was for the CEM cells, there was good agreement with the predictions of the computer simulation. DNBS had little or no effect on the retention and detection of gelsolin, but improved the detection of vimentin. How fixation in the presence of DNBS increases the mean fluorescence of anti-vimentin reactive cells is not known. Because the anti-vimentin was a monoclonal antibody, it is not due to an unmasking of epitopes by DNBS that leads to the binding of other antibody specificities. Instead, this result suggests DNBS may improve access to vimentin in regions of the cell not accessible to antibodies when DNBS is not present during fixation, or that DNBS increases the amount of vimentin retained within fixed cell. Whether it is improved access of antibodies or improved retention of antigen, these results confirmed the added benefit of fixation in the presence of DNBS for some antigens and extended the validity of the computer model to include the behavior of these antigens in peripheral blood cells.

These results show that the combination of active ingredients of the present invention that most improved fixation and detection for each individual antigen could not be predicted in advance. Each individual active ingredient did not influence all antigens in the same way. Detection of gelsolin was most improved by increasing the DMSO concentration; whereas, detection of cytoplasmic CD3 and vimentin was most improved by increasing the detergent concentration. Vimentin and cytoplasmic CD3 detection was improved by increasing the concentration of DNBS, but gelsolin detection was not influenced significantly by DNBS. It is reasonable to speculate, based on these results, that there will be other antigens, either cytoplasmic or cell surface, the fixation of which will be more strongly influenced by certain of the active ingredients over others. However, it remains impossible to anticipate whether an

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antigen will be preserved or destroyed after having been fixed. It is also impossible to anticipate which of the active ingredients will be the dominant ingredient for preserving antigenic structure after fixation. This data further establishes that although staining of cytoplasmic antigens takes place even in the absence or at low concentrations of the various ingredients, immunostaining is improved in many cases by the use of the DNBS and increasing the concentration of one or more of the other active ingredients.

TABLE E3-1

COMPUTER GENERATED STATISTICAL EXPERIMENTAL DESIGN.				
RUN NUMBER	PERCENT FORM- ALDEHYDE	MILLI- MOLES DNBS	PER- CENT DMSO	PERCENT DETERGENT
1	0.70	38.0	4.5	0.07
2	1.00	25.0	4.5	0.10
3	0.85	25.0	4.5	0.07
4	0.85	25.0	1.0	0.04
5	0.85	38.0	4.5	0.10
6	0.85	25.0	4.5	0.07
7	0.70	25.0	1.0	0.07
8	0.85	25.0	8.0	0.10
9	0.70	12.0	4.5	0.07
10	0.85	38.0	8.0	0.07
11	0.70	25.0	8.0	0.07
12	0.70	25.0	4.5	0.10
13	0.85	38.0	1.0	0.07
14	0.70	25.0	4.5	0.04
15	0.85	12.0	4.5	0.10
16	0.85	25.0	1.0	0.10
17	1.00	25.0	1.0	0.07
18	0.85	12.0	1.0	0.07
19	0.85	12.0	8.0	0.07
20	1.00	12.0	4.5	0.07
21	1.00	25.0	4.5	0.04
22	0.85	12.0	4.5	0.04
23	0.85	25.0	4.5	0.07
24	1.00	25.0	8.0	0.07
25	0.85	25.0	8.0	0.04
26	0.85	38.0	4.5	0.04
27	1.00	38.0	4.5	0.07

TABLE E3-2

EFFECT OF DNBS CONCENTRATION DURING FIXATION ON DETECTION OF CYTOPLASMIC ANTIGENS IN LYMPHOCYTES.				
ANTIBODY	LIVE CELLS	LYMPHOCYTES FIXED USING		
		NO DNBS	19.6 mM DNBS	38.0 mM DNBS
Percent Positive:				
Anti-gelsolin	0.45	81.31	81.55	84.30
Anti-vimentin	0.31	89.66	86.82	90.17
Mean Fluorescence:				
Anti-gelsolin	NA	18.91	20.24	21.82
Anti-vimentin	NA	100.51	150.35	187.82

NA = Not applicable.

## EXAMPLE 4

## Utility of Different Detergents

Different detergents have been used in the preparation of the fixative composition of the present invention. The detergents were not only different in composition, but represent

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distinct classes of compounds. The fixative of the present invention was prepared as has been previously described, except in this experiment, the detergent and the detergent concentration were varied. The detergents used were polyoxyethylene ether W-1 (Polyox), polyoxyethylenesorbitans monolaurate (Tween 20), monopalmitate (Tween 40), monooleate (Tween 80) and polyoxyethylene 23 lauryl ether (Brij 35). Other detergents that work well include Nonidet P-40, Triton X-100, sodium deoxycholate and saponin.

In the present example, whole blood from a healthy donor was either resuspended in PBS/S or fixed with an equal volume of fixative reagent for 30 minutes at room temperature. All red blood cells from either fixed or live cell populations were removed by ammonium chloride lysis and the white blood cells washed twice by centrifugation (457×g, 21° C., 8 minutes) in PBS/S. The white blood cells were then immunostained using an indirect immunostain procedure and the antibody anti-vimentin (clone No. V9, Sigma Chemical Co.).

The T-cell tissue culture line CEM was used in addition to peripheral blood cells. Regardless of the fixative being tested, the method of fixation was as follows. CEM cells were pelleted (457×g, 21° C., 8 minutes), then washed twice by centrifugation (457×g, 21° C., 8 minutes) in PBS/S at room temperature. Washed cells were resuspended in PBS/S at a concentration of  $2.5 \times 10^6$  cells/mL. The pool of cells was separated into 1 mL aliquots, then diluted with an equal volume of the appropriate fixative reagent. Cells were mixed and incubated for 30 minutes at room temperature. After 30 minutes, fixed cells were washed twice with 10 mLs of ice cold PBS. All cells were washed once more in PBS/S then resuspended to a concentration of  $3.3 \times 10^6$  cells/mL.

## Indirect Immunostain

Cells were reacted with the mouse monoclonal antibodies anti-gelsolin (clone No. GS-2C4, Sigma Chemical Co.) and anti-vimentin (clone No. V9, Sigma Chemical Co.). Immunostaining of cytoplasmic antigens was done by adding 5  $\mu$ L of Control IgG2a mouse antibody (15  $\mu$ g/mL), or 5  $\mu$ L of anti-gelsolin (diluted 1/100 in PBS/S) or 5  $\mu$ L of anti-vimentin (diluted 1/60 in PBS/S) to 100  $\mu$ L of fixed or live cell suspension. Cells and antibody were incubated for one hour at 0° C., then washed twice in PBS/S using 2 mLs per wash. After the last wash, the supernatant fluid was removed by aspiration, and the cells resuspended in 250  $\mu$ L of goat anti-mouse IgG-FITC conjugate (F(ab')<sub>2</sub>, Sigma Chemical Co.) diluted 1/75 in PBS/S. Cells were again incubated on ice for 60 minutes, then washed three times in PBS/S, using 2 mL per wash. After the last wash, cells were resuspended in 0.5 mL of PBS/S and analyzed on a FACScan flow cytometer.

Immunostained cells, both live and fixed, were analyzed by flow cytometry. For both live and fixed cells, lymphocytes, monocytes and granulocytes were identified solely on the basis of their light scatter properties.

The addition of either polyoxyethylene ether or Brij™ 35 detergents lead to a dose-dependent increase in the percentage of fixed cells that bound anti-vimentin (Table E4-1). This increased cytoplasmic access was seen with all three cell types. More control antibody bound nonspecifically to monocytes and granulocytes than to lymphocytes. The elevated background staining seen with monocytes and granulocytes required the cut off between specific and nonspecific binding to be placed higher with these cell populations. This may explain in part why generally lower

percentages of monocytes and granulocytes appear to have bound antibody, than identically treated lymphocytes. Polyox was more active than Brij™ 35 on a weight basis. No attempt was made to optimize the fixative reagent formulation around these detergents. Polyox, although very active as a cell permeabilization reagent had a profound effect on FSC of granulocytes and monocytes. The polyoxyethylene sorbitan detergents of the Tween series were examined because their structure was similar to polyox but they tend to be gentle detergents. The results of an experiment using CEM cells are shown in Table E4-2. A dose dependent increase in the percentage of cells that reacted with antibodies to the cytoplasmic antigens gelsolin and vimentin was seen with all three detergents. The ability to detect cytoplasmic antigens was dependent on not only the cells being fixed, but also on the concentration of detergent at the time of fixation. Tween 20 gave the greatest percent positive cells, followed by Tween 80 and finally Tween 40.

These data confirm that the immunologic detection of cytoplasmic antigens requires the cells be fixed. Fixation alone will impart only a limited ability to detect cytoplasmic antigens immunologically. The detection of cytoplasmic antigens is greatly improved by detergent treatment of cells; and fixation and detergent treatment may be done simultaneously in a single step. Fixation, followed by a separate permeabilization step is not required for successful retention and detection of cytoplasmic antigens. These data further support that a wide range of detergents may be employed for the purpose of permeabilizing cells. It is reasonable to speculate from these results that the detergent of the preferred embodiment may vary depending upon its intended use. For example, polyox may be the preferred detergent if the intended use requires good cell permeabilization but does not require preserving granulocyte FSC. It is further anticipated that some applications could require a combination of more than one detergent to achieve a desired performance.

TABLE E4-1

CELL TYPE AND THEIR PERCENT POSITIVE STAINING USING ANTI-VIMENTIN			
% DETERGENT	LYMPHO-CYTES	MONOCYTES	GRANULO-CYTES
<b>LIVE CELLS</b>			
0.000	2.70	5.69	6.65
<b>POLYOX</b>			
0.003	25.47	9.65	8.57
0.006	32.68	22.49	12.82
0.018	44.54	39.35	29.51
0.036	67.22	68.85	51.71
<b>BRIJ 35</b>			
0.003	15.34	5.50	7.42
0.006	24.61	8.33	14.07
0.018	20.74	12.46	15.58
0.036	27.57	13.10	22.00

PERCENT DETERGENT IS WEIGHT PER VOLUME.

TABLE E4-2

ABILITY OF DIFFERENT DETERGENTS TO MAKE CEM CELLS PERMEABLE TO ANTIBODY			
TREATMENT	DETERGENT % (W/V)	ANTI-GELSOLIN	ANTI-VIMENTIN
LIVE CELLS	NONE	1.59	1.55
FIXED CELLS	0.000	25.26	27.48
TWEEN 20	0.005	30.24	36.85
	0.010	43.28	37.42
	0.025	80.24	88.07
	0.050	96.67	93.96
	0.005	35.62	52.64
TWEEN 40	0.010	47.87	50.75
	0.025	57.89	65.04
	0.050	68.79	51.93
	0.005	33.92	19.35
	0.010	28.11	42.57
TWEEN 80	0.025	36.99	35.19
	0.050	89.51	90.65

## EXAMPLE 5

## Detection of the Human Immunodeficiency Virus in Infected Cells

Viruses grow within cells. The nucleic acids and proteins that constitute the viral particle are produced by the infected cell and accumulate in the cell. It should be possible to detect the presence of vital proteins in infected cells if the virus is transcriptionally active. The human immunodeficiency virus (HIV) produces many proteins. Some of the proteins regulate vital gene expression and some are structural proteins that make up the core or the envelope of the virus. The protein p24 is a structural protein that HIV infected cells produce in excess. The ability to detect replicating virus in cells may have clinical significance in detecting and monitoring the disease, acquired immunodeficiency syndrome (AIDS), caused by HIV. The virus load in HIV-infected individuals is related to disease progression and prognosis. In the past, and as previously described in the Background of the Invention, virus load has been monitored through the use of culture, polymerase chain reaction (PCR) or the p24 immunoassay. HIV culture and PCR are costly specialized tests, not amenable to most clinical laboratory environments. The p24 assay is often negative in HIV infected individuals, because immune complexes between p24 and the patient's own antibody prevent capture of p24 in commercial assay kits.

To determine if the reagents and methods of the present invention could be used to detect vital p24 within infected cells, HIV infected tissue culture cells and peripheral blood leukocytes from HIV infected individuals were examined. The human tissue culture cell line H9 is capable of supporting the growth of HIV. Uninfected H9 cells were obtained from the National Institutes of Health's AIDS Research and Reference Reagent Program. H9 cells, persistently infected with HIV, were obtained from the American Type Culture Collection. Uninfected and persistently infected H9 cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum. Cultures were passed at least once per week. Blood from three individuals known to be infected with HIV was obtained and stored at room temperature in EDTA for less than 24 hours before being fixed. After fixation, the cells were frozen at -20° C. until thawed for an HIV p24 assay.



The fixative reagent was prepared as a 2× concentrate intended to be diluted with an equal volume of the cell suspension to be fixed. The 2× fixative reagent contained 1.44% formaldehyde, 73.0 mM DNBS, 15.0% DMSO and 50 μM (0.006%) NP40 detergent in phosphate buffered saline pH 7.2. The fixative was stored in amber glass bottles at room temperature until needed; usually less than 5 days. Uninfected and persistently infected H9 cells were pelleted (457×g, 21° C., 8 minutes), then washed once by centrifugation (457×g, 21° C., 8 minutes) in PBS at room temperature. Washed cells were resuspended in PBS at a concentration of approximately  $1 \times 10^6$  cells/mL. Cells were then diluted with an equal volume of fixative reagent. Cells were mixed and incubated for 60 minutes at room temperature. After 60 minutes, fixed cells were washed twice with 10 mLs of ice cold PBS supplemented with 10% fetal bovine serum and 2% human AB serum (PBS/S), then resuspended to a concentration of  $1 \times 10^6$  cells/mL.

Whole blood from a non-AIDS related hospital patient and each of three donors known to be infected with HIV was mixed for 60 minutes with an equal volume of fixative reagent. After 60 minutes at room temperature, fixed blood was washed twice in ice cold PBS/S. All red blood cells were removed by ammonium chloride lysis. The white blood cells were then immunostained using an indirect immunostain procedure.

#### Indirect Immunostain

200 μL of cell suspension was incubated with 20 μL of a mouse monoclonal antibody to HIV p24 (9AIB2, Ortho Diagnostic Systems) diluted 1:40 in PBS/S, or a rabbit polyclonal anti-p24 antibody (Chiron, Emeryville, Calif.) diluted 1:40 in PBS/S. Cells and antibody were incubated for one hour at 0° C., then washed twice in PBS/S. After the last wash, the supernatant fluid was removed by aspiration, and the cells resuspended in 200 μL of either goat anti-mouse IgG-FITC conjugate (F(ab)<sub>2</sub>, Sigma Chemical Co.) diluted 1/75 in PBS/S or goat anti-rabbit IgG-FITC conjugate (F(ab)<sub>2</sub>, Sigma Chemical Co.). Cells were again incubated on ice for 60 minutes, then washed three times in PBS/S. Cells used as negative controls were incubated with IgG2a control mouse antibody, normal rabbit serum, or no antibody at all. The cells were washed then incubated with goat anti-mouse-IgG-FITC or goat anti-rabbit-IgG-FITC. After the last wash, cells were resuspended in 1 mL of PBS/S and analyzed on a Cytofluorograph C50 flow cytometer.

The ability to detect HIV p24 in infected cells was demonstrated unequivocally using uninfected H9 and persistently infected H9 tissue culture cells (FIGS. 4a, 4b, 5a, and 5b). FIGS. 4a and 4b are uninfected cells reacted with anti-p24 monoclonal antibody. In FIG. 4a, the X-axis is FSC or cell size and the Y-axis is the fluorescence intensity of the anti-p24 staining. The data is presented as a contour plot. A contour plot is similar to the three-dimensional representations of data presented elsewhere in this application. In the case of a contour plot, the Z-axis or cell number is perpendicular to the plane of the paper. The observer views the data as if looking straight down on a "mountain" created by a cluster of events. As with geological relief maps, the contour lines represent slices through a peak at regular intervals. FIG. 4b is a histogram plotting the number of cells versus the relative fluorescence intensity; expressed as a percent of the maximum intensity. The log of the mean fluorescence intensity for uninfected H9 cells was 0.75. Only 1.2% of the H9 cells had a fluorescence intensity greater than 1.0. However, as seen in FIGS. 5a and 5b, when persistently HIV infected

cells were treated with anti-p24 there was a marked increase in fluorescence intensity. The log of the mean fluorescence intensity for infected H9 cells was 3.27; and 86.2% of the H9 cells had a fluorescence intensity greater than 1.0. The binding of anti-p24 to p24 within the infected cells was also evidenced by the fluorescence intensity histogram, shown in FIG. 5b, shifting to the right when infected cells were used.

FIGS. 6a, 6b, 6c and 6d show the results when blood from HIV infected individuals and an uninfected control individual was tested. Side scatter was plotted on the X-axis and the fluorescence intensity of anti-p24 binding was plotted on the Y axis. Side scatter alone was not able to resolve monocytes from granulocytes in this experiment. This was most likely due to the cells having been frozen and thawed before being assayed. When patient cells had not been frozen and thawed prior to being used, light scatter could differentiate fixed lymphocytes, monocytes and granulocytes of HIV infected and AIDS patients as well as it could for uninfected individuals. FIG. 6a shows the result for a control HIV uninfected hospital patient. There is no evidence of anti-p24 antibody binding to cells in either the lymphocyte cluster on the left or in the monocyte-granulocyte cluster on the right. FIG. 6b shows the result from an individual classified as CDC stage III disease and taking the drug Zidovudine (AZT). The individual was asymptomatic at the time of blood draw. Note the distribution of the lymphocyte cluster is skewed upward on the Y-axis compared to the control cells. A number of lymphocytes bound a low level of anti-p24 in this patient. In addition, a cluster of a small number of brightly staining monocytes were clearly evident in this patient. FIG. 6c shows the results of an individual whose disease had progressed to CDC stage IV. This individual was suffering from oral candidiasis at the time the blood was drawn. In this patient the lymphocyte cluster was completely negative for anti-p24 binding. Granulocytes too seemed free of viral proteins but there was a striking involvement of the patient's monocytes. Finally, FIG. 6d presents the results of a patient with full blown AIDS. This individual had developed a peripheral neuropathy as a consequence of drug treatment and had to be removed from anti-retroviral chemotherapy. It was evident that numerous lymphocytes, monocytes and granulocytes bound anti-p24 antibody in this patient. Many cells bound so much anti-p24, their fluorescence was off scale.

These results demonstrate the methods and reagents of the present invention may be used for the immunologic detection of viral proteins within infected cells. The surface differentiation markers on cells are preserved by the methods and reagents of the present invention, while also providing antibody access to internal antigens. It is possible to prepare a mixture of antibodies that contains an antibody to a cell surface differentiation marker with antibodies to internal antigens such as HIV structural proteins. For example anti-CD4-PE could be mixed with anti-p24-FITC. If such a mixture of antibodies was reacted with cells from an HIV infected individual, and examined on a flow cytometer, it would be possible to determine not only how many CD4 positive cells the individual had, but also how many of the CD4 positive cells were expressing viral p24 antigen. Such information may have prognostic or therapeutic value in the clinical management of HIV infection. It is obvious to those skilled in the art that this principle could be extended to other intrinsic or extrinsic cellular antigens, and is in no way limited to HIV or extrinsic pathogens.



## Virus Inactivation Studies

## Inactivation Of Rabbitpox virus

The fixative formulation and fixation methods of the present invention were tested for their ability to inactivate live virus. Three viruses were chosen for study; Rabbitpox, Simian virus 40 (SV40) and Human Immunodeficiency Virus type 1 (HIV-1). For these studies the fixative contained; 1.44% (w/v) formaldehyde, 73 mM dinitrobenzene sulfonic acid, 50 uM Nonidet P40 and 15% (v/v) dimethyl sulfoxide.

The Utrecht strain of Rabbitpox (ATCCVR-157) was used as the target virus. Vero cells (ATCC CCL 81) were used as the host cell for growing the virus. Vero cells were infected with Rabbitpox virus and incubated for 24 hours. Approximately  $3 \times 10^7$  infected cells were washed, then resuspended in 1.0 mL of cell free Rabbitpox virus. The mixture was then diluted with 1.0 mL of fetal bovine serum to make the mixture 50% serum. A 0.1 mL sample was removed to test the total viral burden; this sample was labeled "Vital Load". The rest of the virus/cell suspension (1.9 mL) was mixed with 1.9 mL of fixative. A 0.1 mL sample was immediately removed and diluted 1:30 in EMEM+5% Tryptose Phosphate Broth (TSB). This represented the T=0 sample. The rest of the suspension was incubated 30 minutes at room temperature. At 30 minutes, a 0.1 mL sample was again taken and diluted 1:30 in EMEM+5% TSB, this represented the T=30 sample. Both the T=0 and T=30 samples were centrifuged to pellet cells. The supernatants were removed and kept on ice while the cell pellets were subjected to two rounds of freezing and thawing to lyse cells and release cell associated virus. The supernatant fluids were recombined with the lysed cell pellets and clarified by centrifugation to remove cell debris. The resulting supernatant fluids containing free and released virus were serially diluted and 0.1 mL samples inoculated into dishes containing uninfected Vero cells grown to confluence. The Vero cell cultures were incubated for 90 minutes at 36° C. to allow any live virus present to infect the cells. The cultures were then washed free of the vital inoculum, overlaid with medium containing agarose and incubated. Growth of the virus was quantitated by counting the number of plaques formed. All cultures were set up with appropriate positive and negative controls; and the results are expressed as the number of plaque forming units per mL (PFU/mL).

TABLE E6-1

Rabbitpox Inactivation						
SAMPLE	DI-LUTION	PLAQUES/ DISH		MEAN		PFU/mL
NEG- ATIVE	None	0,	0,	0	0	0
POSITIVE	$10^{-5}$	133,	128,	146	136	$1.36 \times 10^6$
	$10^{-6}$	10,	12,	10	11	$1.1 \times 10^6$
	$10^{-7}$	1,	3,	4	3	$3 \times 10^6$
Mean Viral Titer = $1.2 \times 10^6$ PFU/mL.						
VIRAL LOAD	$10^{-5}$	451,	422,	434	436	$4.36 \times 10^7$
	$10^{-6}$	55,	59,	50	55	$5.5 \times 10^7$
	$10^{-7}$	8,	8,	11	9	$9 \times 10^7$
Mean Viral Titer = $4.9 \times 10^7$ PFU/mL.						
T = 0	$10^{-5}$	348,	309,	304	320	$3.20 \times 10^6$
	$10^{-6}$	32,	41,	22	32	$3.2 \times 10^6$
	$10^{-7}$	2,	7,	9	6	$6 \times 10^6$
	$10^{-8}$	0,	1,	1	0.7	$7 \times 10^6$
Mean Viral Titer = $3.2 \times 10^6$ PFU/mL.						

TABLE E6-1-continued

Rabbitpox Inactivation						
SAMPLE	DI-LUTION	PLAQUES/ DISH		MEAN		PFU/mL
T = 30	1:30	0,	0,	0	0	0
	$10^{-2}$	0,	0,	0	0	0
	$10^{-3}$	0,	0,	0	0	0
	$10^{-4}$	0,	0,	0	0	0
10	$10^{-5}$	0,	0,	0	0	0
	$10^{-6}$	0,	0,	0	0	0
Mean Viral Titer = less than 90 PFU/mL.						

The total viral burden of the sample treated with fixative was  $4.9 \times 10^7$  PFU/mL (Table E6-1). More than a log of virus was inactivated immediately on contact with the fixative as seen by the reduction to  $3.2 \times 10^6$  PFU/mL in the T=0 sample. After 30 minutes of fixative treatment, no viable Rabbitpox virus could be detected. It is significant that even cell associated virus was killed; suggesting a viro-cydal concentration of fixative was able to penetrate Vero cells.

## Inactivation of SV40 virus

The PA-57 strain of SV40 was used as the target virus. CV-1 cells (ATCC CCL 70) were used as the host cell for growing the virus. CV-1 cells were infected with SV40 virus and incubated for 72 hours. Approximately  $2 \times 10^7$  infected cells were washed, then resuspended in 1.0 mL of cell free SV40 virus. The mixture was then diluted with 1.0 mL of fetal bovine serum to make the mixture 50% serum. A 0.1 mL sample was removed to test the total viral burden; this sample was labeled "Vital Load". The rest of the virus/cell suspension (1.9 mL) was mixed with 1.9 mL of fixative. A 0.1 mL sample was immediately removed and diluted 1:30 in EMEM+5% TSB. This represented the T=0 sample. The rest of the suspension was incubated 30 minutes at room temperature. At 30 minutes, a 0.1 mL sample was again taken and diluted 1:30 in EMEM+5% TSB, this represented the T=30 sample. Both the T=0 and T=30 samples were centrifuged to pellet cells. The supernatants were removed and kept on ice while the cell pellets were subjected to two rounds of freezing and thawing to lyse cells and release cell associated virus. The supernatant fluids were recombined with the lysed cell pellets and clarified by centrifugation to remove cell debris. The resulting supernatant fluids containing free and released virus were serially diluted and 0.1 mL samples inoculated into dishes containing uninfected CV-1 cells grown to confluence. The CV-1 cell cultures were incubated for 90 minutes at 36° C. to allow any live virus present to infect the cells. The cultures were then washed free of the viral inoculum, overlaid with medium containing agarose and incubated. Growth of the virus was quantitated by counting the number of plaques formed. All cultures were set up with appropriate positive and negative controls; and the results are expressed as the number of plaque forming units per mL (PFU/mL).

TABLE E6-2

SV40 Inactivation						
SAMPLE	DI-LUTION	PLAQUES/ DISH		MEAN		PFU/mL
NEG- ATIVE	None	0,	0,	0	0	0
POSITIVE	$10^{-5}$	65,	69,	82	72	$7.2 \times 10^7$
	$10^{-6}$	7,	11,	7	8	$8 \times 10^7$
Mean Viral Titer = $7.2 \times 10^7$ PFU/mL.						
VIRAL	$10^{-5}$	76,	66,	76	73	$7.3 \times 10^7$

TABLE E6-2-continued

SV40 Inactivation					
SAMPLE	DILUTION	PLAQUES/ DISH			PFU/mL
LOAD	$10^{-6}$	3	4	10	$9 \times 10^7$
Mean Viral Titer = $7.3 \times 10^7$ PFU/mL					
T = 0	$10^{-5}$	153	162	183	$1.66 \times 10^8$
	$10^{-6}$	28	14	26	$2.3 \times 10^8$
Mean Viral Titer = $2.0 \times 10^8$ PFU/mL					
T = 30	$10^{-5}$	121	98	77	$9.9 \times 10^5$
	$10^{-6}$	18	9	15	$1.4 \times 10^6$
	$10^{-7}$	0	0	0	0
	$10^{-8}$	0	0	0	0
Mean Viral Titer = $1.2 \times 10^6$ PFU/mL					

The fixative inactivated SV40, but to a lesser extent than the Rabbitpox virus. Concentrations of fixative components, incubation time, and the like could be adjusted to enhance the virus inactivation capabilities. A maximum of one log of virus was inactivated by a 30 minute treatment with the fixative formulation tested.

#### Inactivation of HIV-1 virus

The HTLV-III-B strain of HIV-1 was used as the target virus. MT-4 cells were used as the host cell for growing the virus. MT-4 cells were infected with HIV-1 virus and incubated for 48 hours. Approximately  $8 \times 10^6$  cells were pelleted, then resuspended in 0.8 mL of cell free HIV-1 virus. 0.4 mLs of this mixture was then diluted with 0.4 mL of fetal bovine serum to make the mixture 50% serum. A 0.1 mL sample was removed to test the total viral burden; this sample was labeled "Viral Load". The rest of the virus/cell suspension (0.7 mL) was mixed with 0.7 mL of fixative. A 0.1 mL sample was immediately removed and diluted 1:300 in RPMI 1640+10% FBS. This represented the T=0 sample. The rest of the suspension was incubated 30 minutes at room temperature. At 30 minutes, a 0.1 mL sample was again taken and diluted 1:300 in RPMI 1640+10% FBS, this represented the T=30 sample. Both the T=0 and T=30 samples were serially diluted and 0.1 mL samples inoculated into dishes containing 1.0 mL of uninfected MT-4 cells. The MT-4 cell cultures were not washed free of the inoculum, but cultures were fed twice per week by removing 1.0 mL of medium and replacing it with a fresh 1.0 mL. Cultures were examined on days 7, 14 and 28 post inoculation for the presence of cytopathic effect (CPE). Cytopathic effect is a morphologic change to an infected cell that occurs as a result of viral growth. In addition, supernatant fluids from 7, 14 and 28 day cultures were collected and assayed for the presence of HIV-1 specific viral p24 protein.

All cultures were set up with appropriate positive and negative controls; and the results are expressed as the percentage of inoculated wells containing CPE or the percentage of wells positive for p24 protein. The tissue culture infectious dose<sub>50</sub> (TCID<sub>50</sub>) per mL was calculated using the formula  $TCID_{50} = A - (S/100 - 0.5) \times B$ ; where  $A = \text{Log}_{10}$  of the highest concentration inoculated,  $S = \text{sum of the percentage positive at each dilution}$  and  $B = \text{Log}_{10}$  of the dilution factor.

TABLE E6-3

HIV-1 Inactivation, CPE				
SAMPLE	DILUTION	% OF WELLS SHOWING CPE		
		DAY 7	DAY 14	DAY 28
NEGATIVE	None	0	0	0
POSITIVE	$10^{-5}$	0	100	100
	$10^{-6}$	0	50	100
	$10^{-7}$	0	50	100
	$10^{-8}$	0	0	25
	$10^{-9}$	0	0	0
TCID <sub>50</sub> /mL =		$10^{5.5}$	$10^{7.5}$	$10^{8.75}$
VIRAL LOAD	$10^{-5}$	100	100	100
	$10^{-6}$	25	100	100
	$10^{-7}$	0	25	100
	$10^{-8}$	0	25	100
	$10^{-9}$	0	0	0
TCID <sub>50</sub> /mL =		$10^{6.75}$	$10^{8.0}$	$10^{9.5}$
T = 0	$1.3 \times 10^{-3}$	75	100	100
	$1.3 \times 10^{-4}$	0	100	100
	$1.3 \times 10^{-5}$	0	75	100
	$1.3 \times 10^{-6}$	0	50	75
	$1.3 \times 10^{-7}$	0	0	0
TCID <sub>50</sub> /mL =		$10^{4.75}$	$10^{7.25}$	$10^{7.75}$
T = 30	$1.3 \times 10^{-3}$	0	0	100
	$1.3 \times 10^{-4}$	0	0	100
	$1.3 \times 10^{-5}$	0	0	25
	$1.3 \times 10^{-6}$	0	0	0
	$1.3 \times 10^{-7}$	0	0	0
TCID <sub>50</sub> /mL =		$<10^{3.0}$	$<10^{3.0}$	$10^{5.25}$

TABLE E6-4

HIV-1 Inactivation, Viral p24 Protein				
SAMPLE	DILUTION	% OF WELLS POSITIVE FOR p24		
		DAY 7	DAY 14	DAY 28
NEGATIVE	None	0	0	0
POSITIVE	$10^{-5}$	100	ND <sup>a</sup>	ND
	$10^{-6}$	100	100	100
	$10^{-7}$	100	100	100
	$10^{-8}$	100	100	100
	$10^{-9}$	100	100	100
TCID <sub>50</sub> /mL =		$10^{10.5}$	$10^{10.5}$	$10^{10.5}$
VIRAL LOAD	$10^{-5}$	ND	ND	ND
	$10^{-6}$	100	100	100
	$10^{-7}$	100	100	100
	$10^{-8}$	100	100	100
	$10^{-9}$	100	100	100
TCID <sub>50</sub> /mL =		$10^{10.5}$	$10^{10.5}$	$10^{10.5}$
T = 0	$1.3 \times 10^{-3}$	100	100	100
	$1.3 \times 10^{-4}$	100	100	100
	$1.3 \times 10^{-5}$	100	100	100
	$1.3 \times 10^{-6}$	100	100	100
	$1.3 \times 10^{-7}$	100	75	100
TCID <sub>50</sub> /mL =		$10^{9.0}$	$10^{8.75}$	$10^{9.0}$
T = 30	$1.3 \times 10^{-2}$	100	100	100
	$1.3 \times 10^{-3}$	100	25	100
	$1.3 \times 10^{-4}$	0	25	25
	$1.3 \times 10^{-5}$	0	25	0
	$1.3 \times 10^{-6}$	0	0	0
TCID <sub>50</sub> /mL =		$10^{5.0}$	$10^{4.75}$	$10^{5.25}$

Treatment with the fixative for 30 minutes at room temperature inactivated between 4 and 5 logs of TCID<sub>50</sub> of HIV-1. It is noteworthy that virus not inactivated by the treatment was impaired significantly with regard to its growth kinetics. As can be seen from Table E6-3, on day 28 there was an estimated TCID<sub>50</sub> =  $10^{5.25}$ /mL in the T=30 sample; yet no CPE was observed with this sample on days 7 and 14. In contrast, dilutions of the Virus Load and T=0

samples that contain comparable amounts of infectious virus ( $10^{-5}$  or  $10^{-6}$ ) did show CPE on days 7 and 14.

### EXAMPLE 7

#### Immunostaining During Fixation

Fixative was prepared with the following formulation: 0.89% (wt/v) formaldehyde; 7.25% (wt/v) DMSO; 32 mM DNBS; 0.0998% (wt/v) Tween 20; 0.2 g/L  $\text{KH}_2\text{PO}_4$ ; 0.2 g/L KCl; 2.16 g/L  $\text{Na}_2\text{HPO}_4 \cdot 7 \text{H}_2\text{O}$  and 4.2 g/L NaCl. Whole blood was collected by venipuncture in K3-EDTA and kept at room temperature until needed, usually less than 2 hours.

To 100  $\mu\text{L}$  aliquots of whole blood was added antibodies to the cell surface antigens CD3 (OKT3), CD2 (OKT11), CD14 (OKM14) and control IgG; or to the cytoplasmic antigen vimentin (clone No. V9, Sigma Chemical Co.). Cells and antibodies were mixed and incubated at room temperature for 20 minutes, then 2 mL of the fixative formulation described above was added to each tube, mixed, and the cell suspension incubated for 40 minutes. After being fixed, cells were pelleted by centrifugation ( $457 \times g$ ,  $21^\circ \text{C}$ ., 8 minutes), the supernatant fluids were removed by aspiration and 3 mL of wash buffer (5% (v/v) Serum, 1.5% (w/v) bovine serum albumin and 0.0055% (w/v) ethylene diamine tetraacetic acid) added to each tube. Tubes were incubated for 10 minutes at room temperature. During this 10 minute incubation, red blood cells but not white blood cells lysed. White blood cells were then pelleted by centrifugation ( $457 \times g$ ,  $21^\circ \text{C}$ ., 8 minutes) and washed once more in 3 mLs of wash buffer. After being washed, cells treated with direct-labeled antibodies (control, OKT3, OKT11 and OKM14) were resuspended in 0.5 mL of PBS+2% formaldehyde and analyzed by flow cytometry. Cells that had been incubated with anti-vimentin were treated for 30 minutes at room temperature with 200  $\mu\text{L}$  of goat-anti-mouse IgG coupled to FITC. These cells were washed two times with 3 mL of wash buffer, then resuspended in 0.5 mL of PBS+2% formaldehyde and analyzed by flow cytometry.

TABLE E7-1

TREATMENT	ANTIBODY	PERCENT POSITIVE CELLS	
		LYMPHO-CYTES	MONOCYTES
No fixation	Control IgG	0.79	0.39
	OKT3	67.92	3.62
	OKT11	77.39	4.12
	OKM14	0.12	82.31
	Anti-vimentin	1.34	6.47
Fixed cells	Control IgG	1.18	0.5
	OKT3	66.19	0.15
	OKT11	77.62	0.99
	OKM14	0.34	88.37
	Anti-vimentin	91.14	95.20

As shown in Table E7-1, there was good agreement between the fixed and unfixed cells in the percentage of lymphocytes and monocytes reactive with antibodies to cell surface components. It is not possible to determine from this experiment how much of the cell surface staining took place during the 20 minute incubation before the addition of the fixative and how much occurred after fixative addition. However, it is reasonable to assume most of the cell surface

reactivity occurred before the fixative was added. In contrast, reactivity with anti-vimentin could only have taken place in the presence of the fixative. Unfixed cells failed to react with anti-vimentin because the antibody could not gain access to the cytoplasmic location of the antigen. Addition of the fixative reagent however, fixed and permeabilized the cells and gave anti-vimentin access to the cytoplasmic antigen. The antigen antibody interaction took place in the presence of the fixative reagent, and the specificity of the reaction was demonstrated by the failure of the control antibody to stain cells.

What is claimed:

1. A method for monitoring human immunodeficiency virus (HIV) infection in a patient so infected, comprising the steps of:

a) contacting a sample of whole blood from said patient with a fixative composition in an amount and for a period of time effective to fix white blood cells present in said whole blood, without substantially destroying the ability of white blood cell antigens and viral components to bind ligands, wherein said fixative composition comprises:

- a first fixative compound selected from the group consisting of 2,4-dinitrobenzene sulfonic acid, 2,4-dinitrobenzoic acid, 2,4-dinitrophenol, and a combination of two or more of these;
- a second fixative compound which is methanol-free, high-grade formaldehyde in a concentration ranging from about 0.1% to about 4%;
- dimethylsulfoxide in a concentration of about 1% (v/v) to about 20% (v/v); and
- a polyoxyethylene sorbitan surfactant in a concentration of about 0.001% to about 0.2% (w/v);

b) isolating white blood cells present in said sample;

c) either concurrently with the contact Step in a) or thereafter, contacting the cells so fixed with at least one antibody to a white blood cell antigen and at least one binding ligand that binds to at least one component from HIV;

d) examining the scattering and fluorescent properties of said cells so fixed with a flow cytometer; and

e) comparing the results of said examination to data obtained in the same manner from patients at various stages of HIV infection.

2. The method of claim 1 wherein said first fixative compound is 2,4-dinitrobenzene sulfonic acid.

3. The method of claim 1 wherein said at least one binding ligand that binds to said component from HIV is anti-p24 antibody.

4. The method of claim 1 wherein said at least one antibody to a white blood cell antigen is an anti-CD4 monoclonal antibody.

5. The method of claim 4 wherein said anti-CD4 monoclonal antibody is labelled with phycoerythrin and said anti-p24 antibody is labelled with FITC.

6. A reagent kit for monitoring HIV load in HIV-infected white blood cells, comprising:

a) a fixative composition which comprises:

- a first fixative compound selected from the group consisting of 2,4-dinitrobenzene sulfonic acid, 2,4-dinitrobenzoic acid, 2,4-dinitrophenol, and a combination of two or more of these;
- a second fixative compound which is methanol-free, high-grade formaldehyde in a concentration ranging from about 0.1% to about 4%;

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- iii) dimethylsulfoxide in a concentration of about 1% (v/v) to about 20% (v/v); and
- iv) a polyoxyethylene sorbitan surfactant in a concentration of about 0.001% to about 0.2% (w/v); and
- b) a binding ligand that binds to an intracellular antigen 5 from HIV.

7. The reagent kit of claim 6 further comprising at least one antibody to a white blood cell surface antigen.

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8. The reagent kit of claim 7 wherein at least one of said antibodies to a white blood cell surface antigen is a monoclonal antibody to CD4 positive T cells.

9. The reagent kit of claim 8 further comprising at least one monoclonal antibody to monocytes.

\* \* \* \* \*

# United States Patent [19]

Lifson et al.

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[54] METHOD OF SELECTIVELY INHIBITING HIV

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[\*] Notice: The portion of the term of this patent subsequent to Jan. 3, 2006 has been disclaimed.

[21] Appl. No.: 179,274

[22] Filed: Apr. 8, 1988

## Related U.S. Application Data

[63] Continuation-in-part of Ser. No. 56,558, May 29, 1987, Pat. No. 4,793,739.

[51] Int. Cl.<sup>4</sup> ..... A61K 37/02; A61K 35/78; A61K 35/80; C07G 7/00

[52] U.S. Cl. .... 424/195.1; 514/8; 514/885

[58] Field of Search ..... 514/8, 12, 21, 885; 424/88, 91, 195.1; 530/370, 379, 806

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[57]

## ABSTRACT

A method of inhibiting HIV replication in and cellular proliferation of HIV-infected cells. The infected cells are exposed to a single-chain ribosome inactivating protein, at a protein concentration and for an exposure period sufficient to produce a substantial reduction in (a) the level of HIV antigen or reverse transcriptase associated with the infected cells, (b) the ratio of viability of infected/uninfected T cells, and/or (c) the ratio of HIV antigen/cellular antigen in infected macrophages. The method is used to treat HIV infection in humans.

20 Claims, 14 Drawing Sheets

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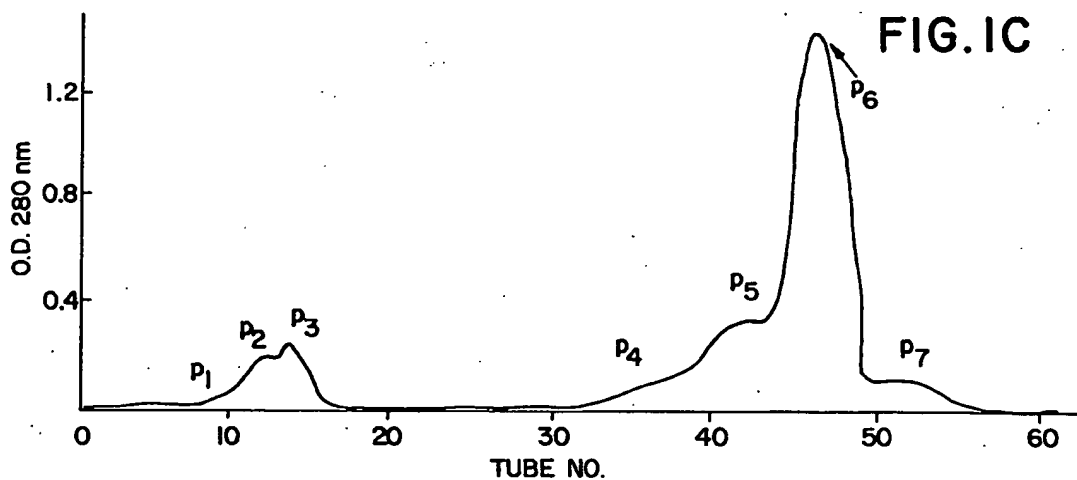
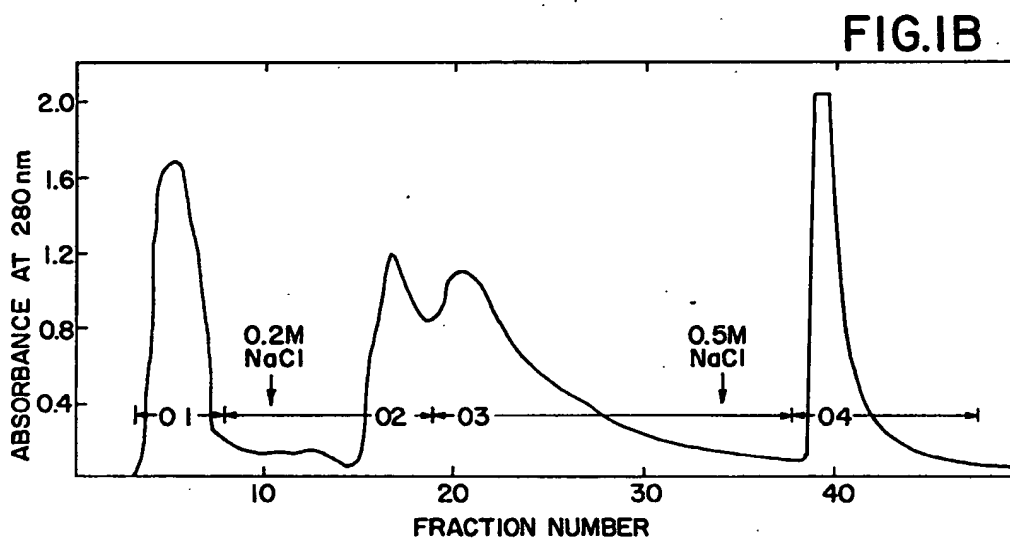
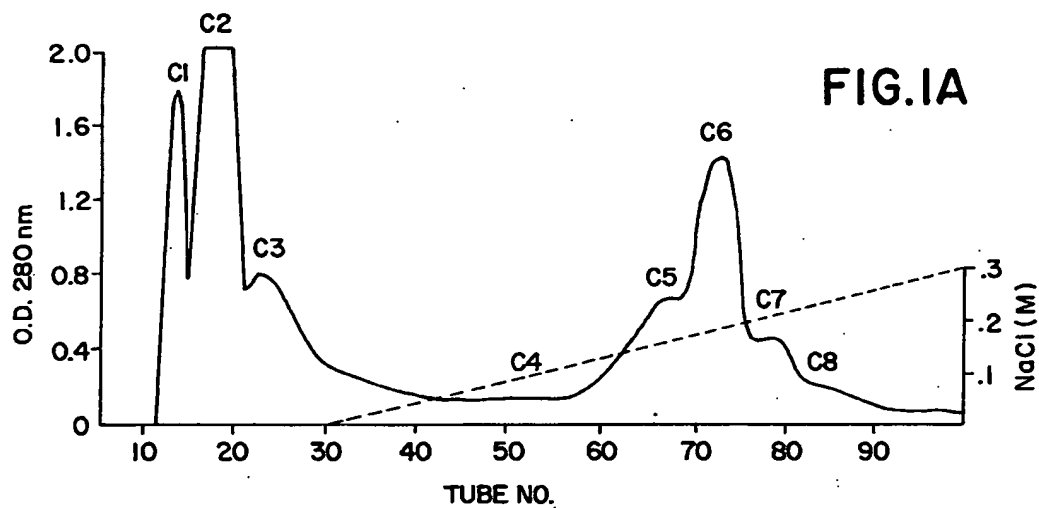




FIG. 2A

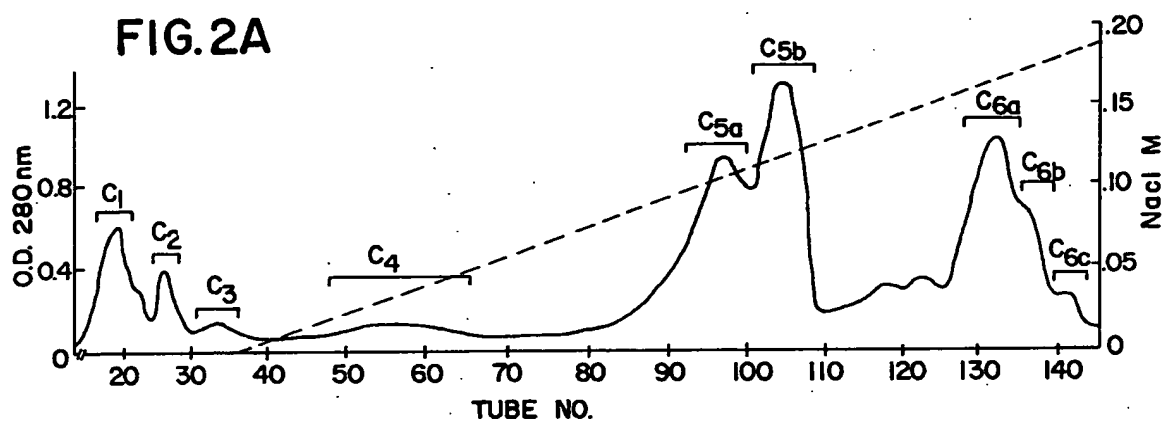


FIG. 2B

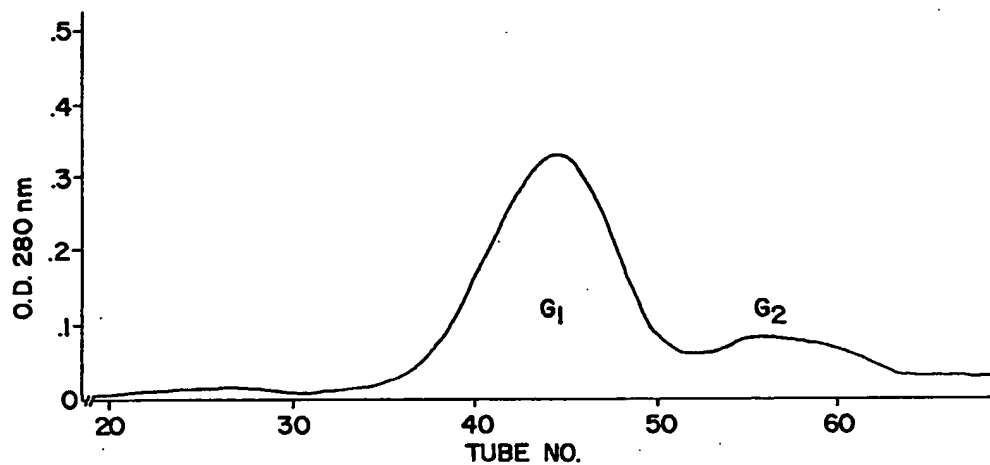
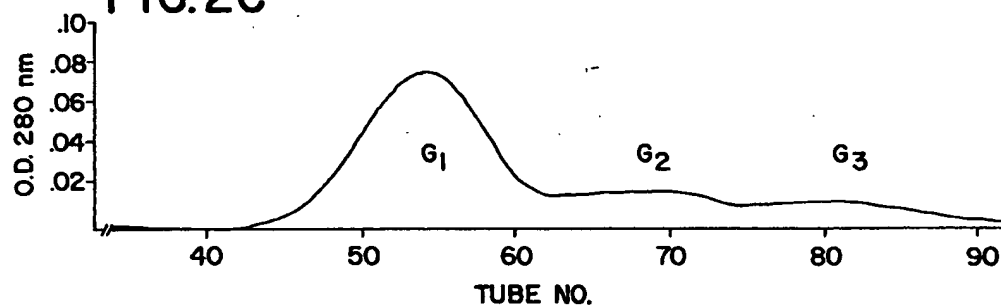


FIG. 2C



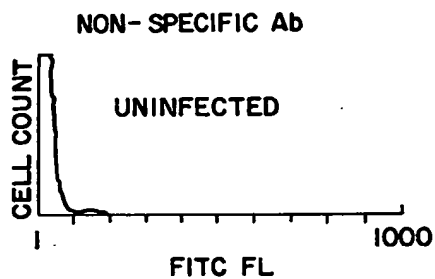


FIG. 3A

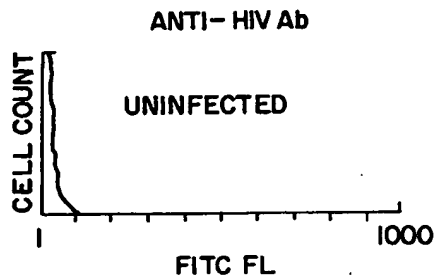


FIG. 3B

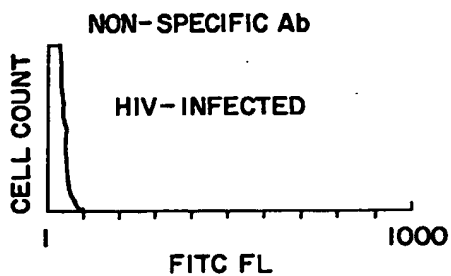


FIG. 3C

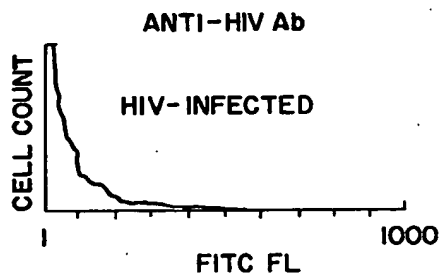


FIG. 3D

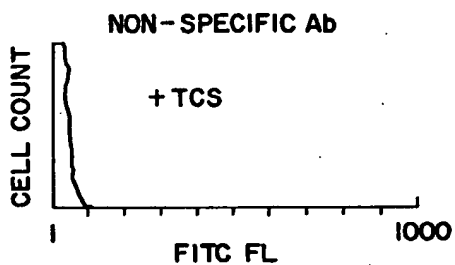


FIG. 3E

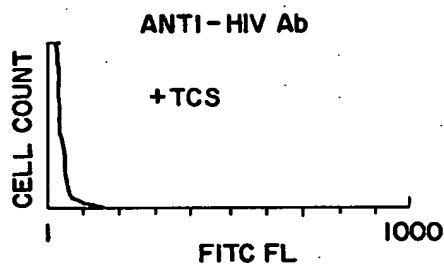


FIG. 3F

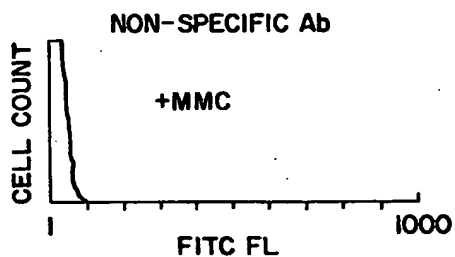


FIG. 3G

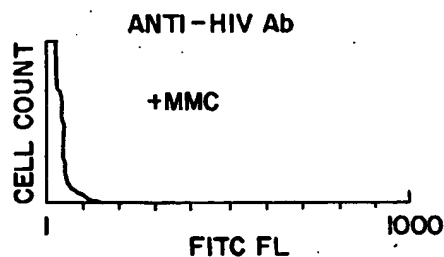
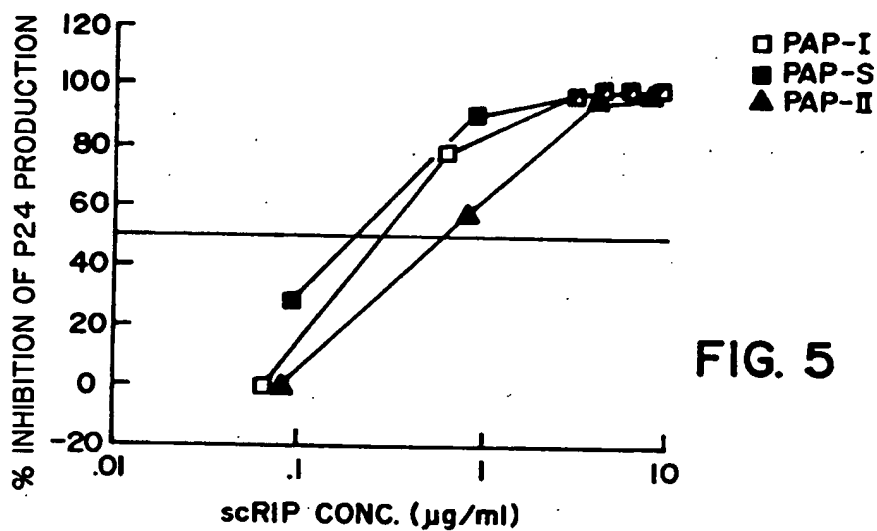
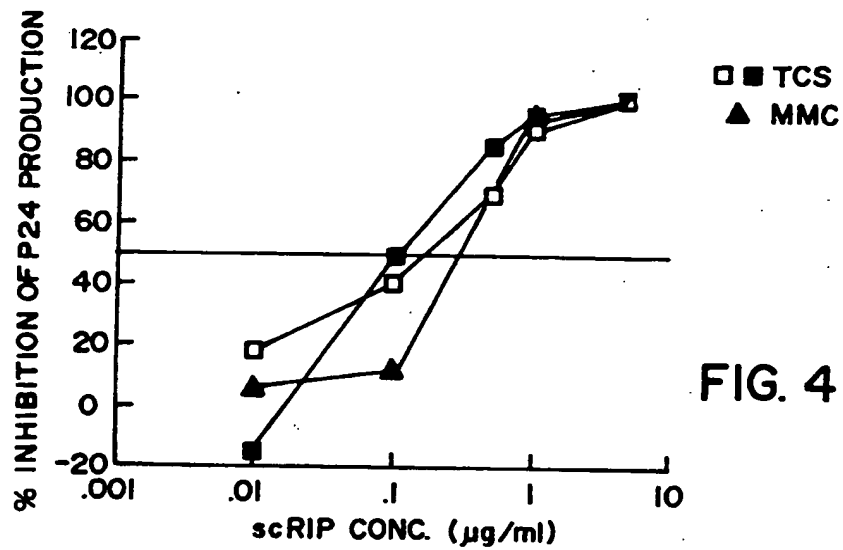


FIG. 3H



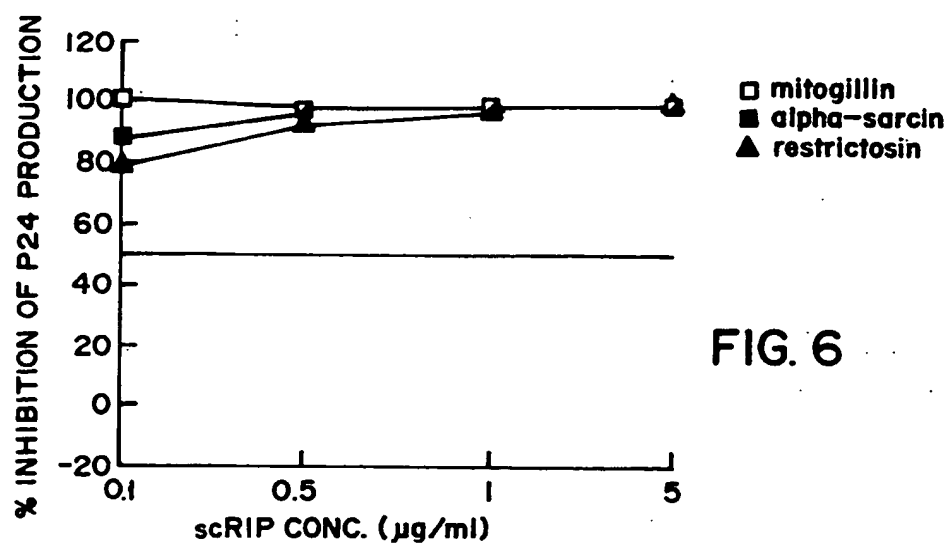


FIG. 6

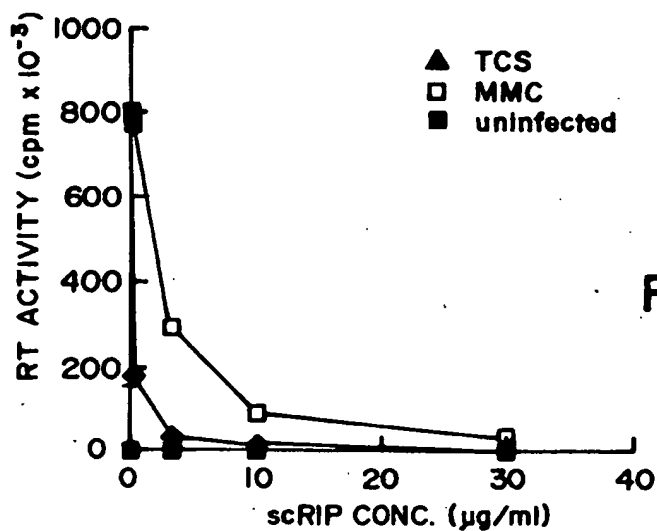
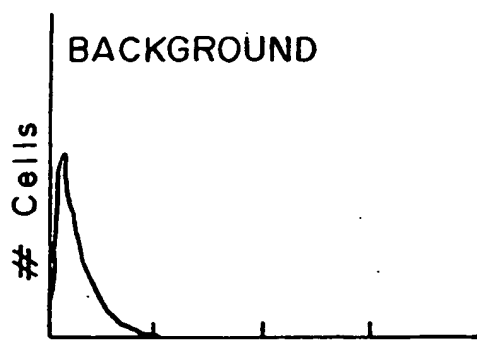
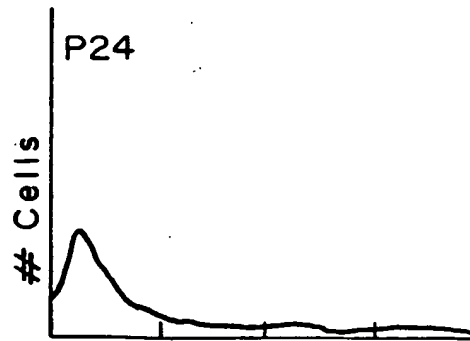


FIG. 7



Fluorescein Units

FIG. 8A



Fluorescein Units

FIG. 8B

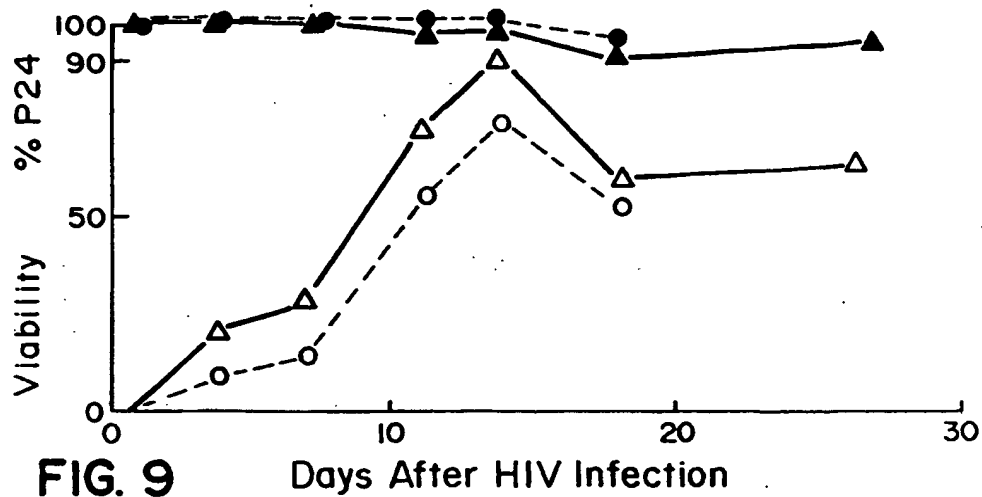


FIG. 9

Days After HIV Infection

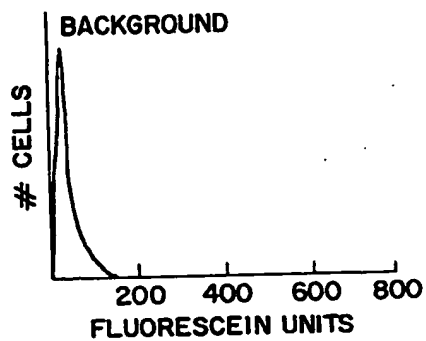


FIG.10A

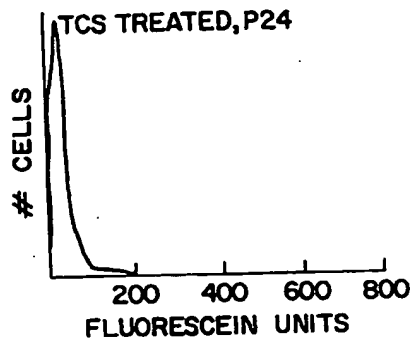


FIG.10C

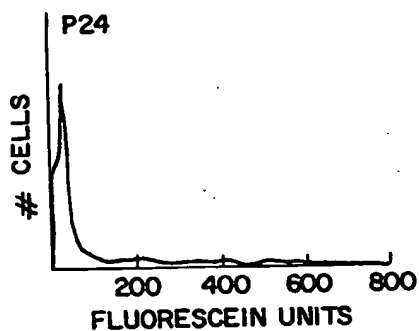


FIG.10B

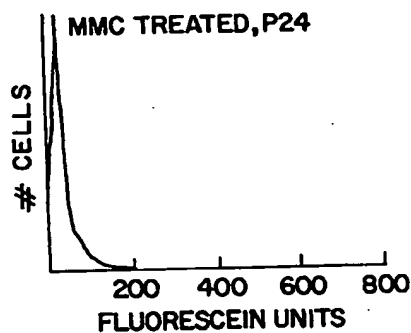


FIG.10D

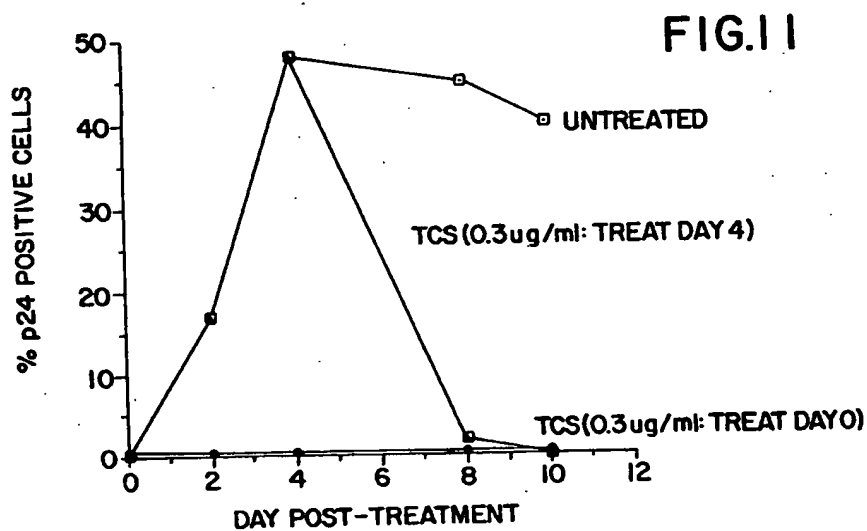


FIG. 12

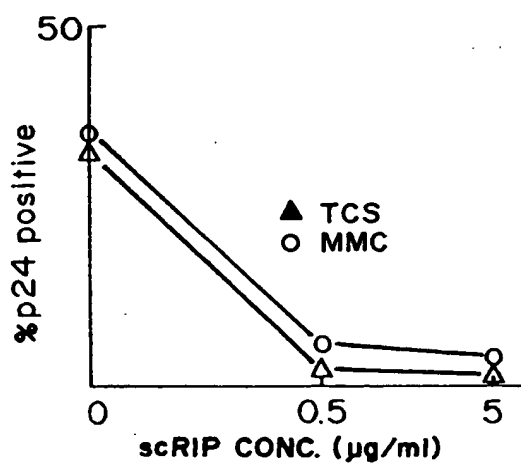
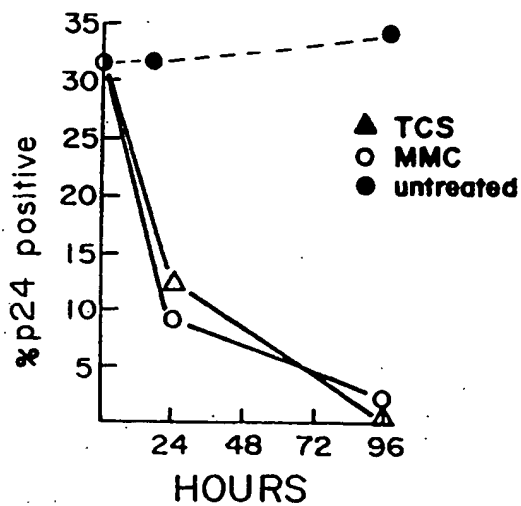


FIG. 13

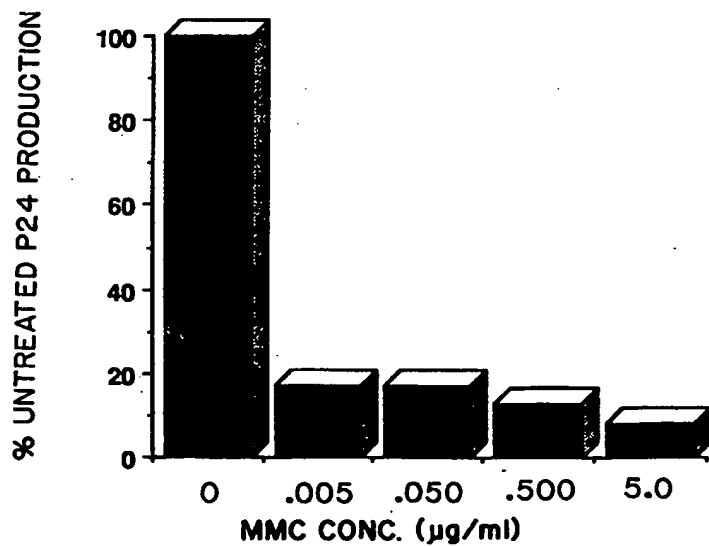


FIG. 14

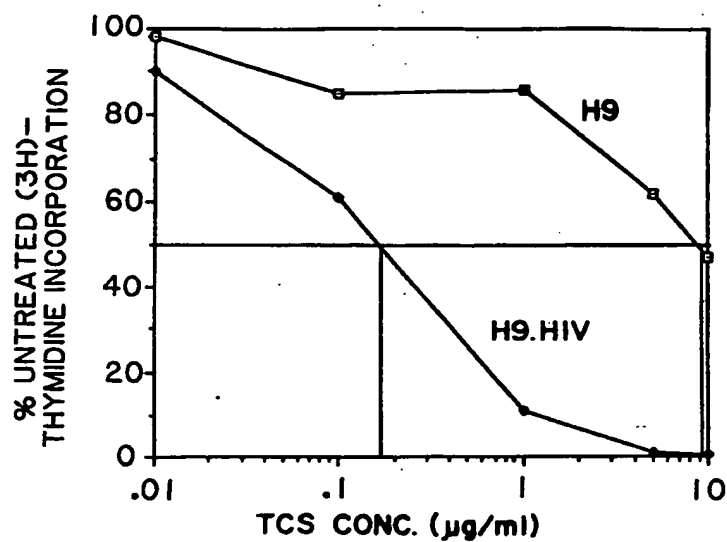


FIG. 15

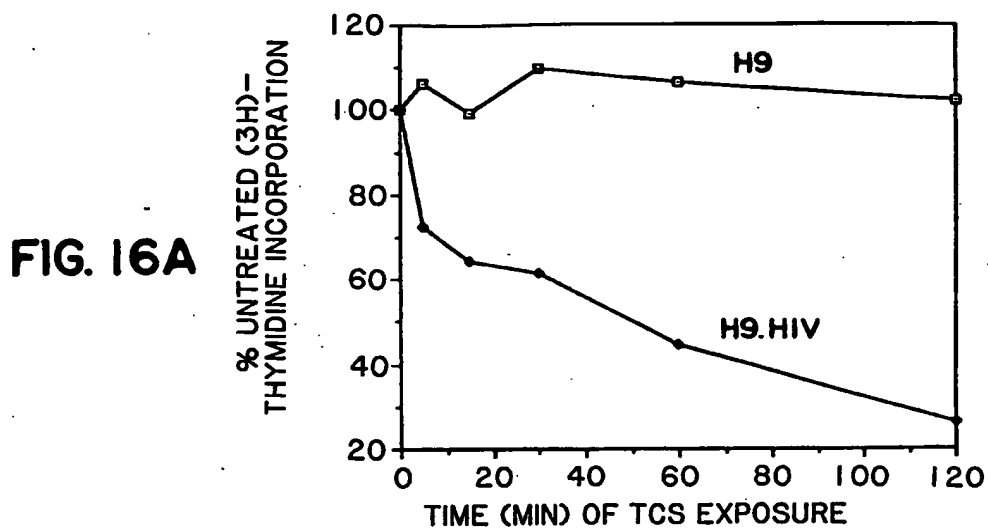


FIG. 16A

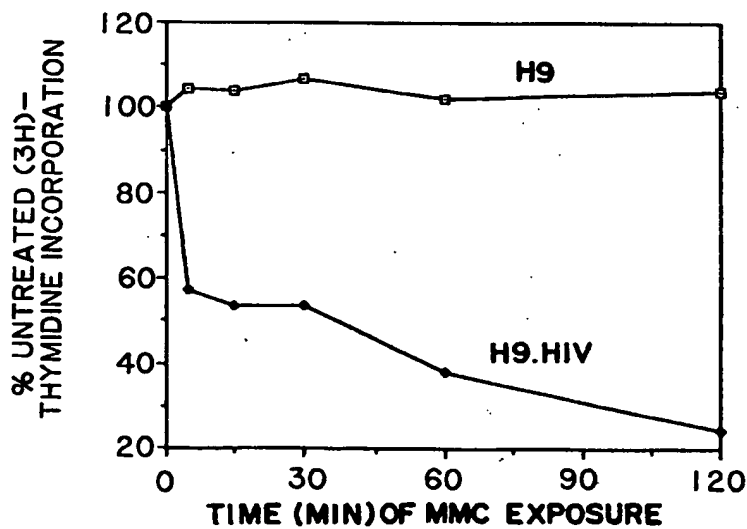


FIG. 16B



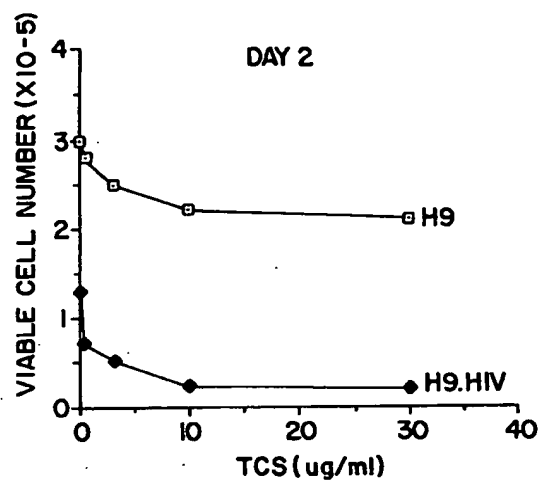


FIG.17A

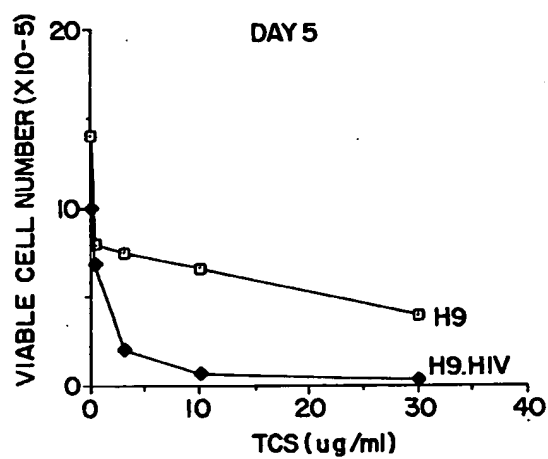


FIG.17B

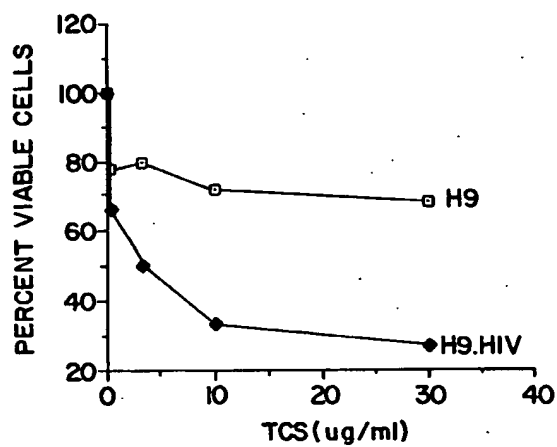


FIG.17C

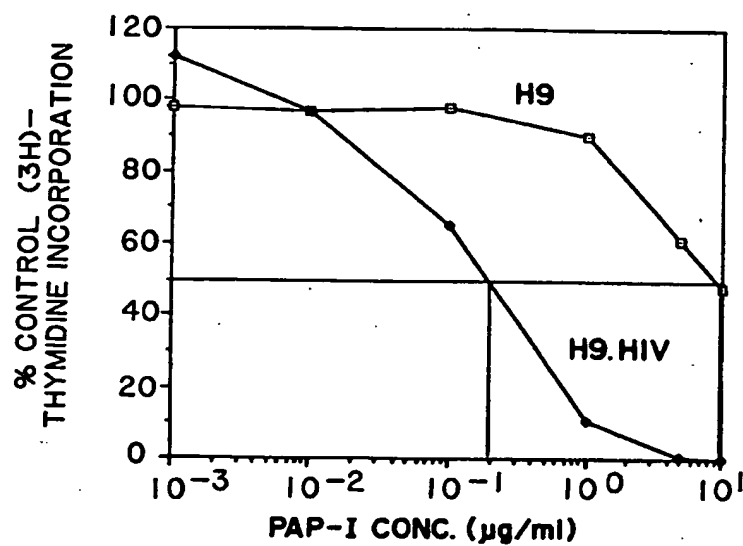
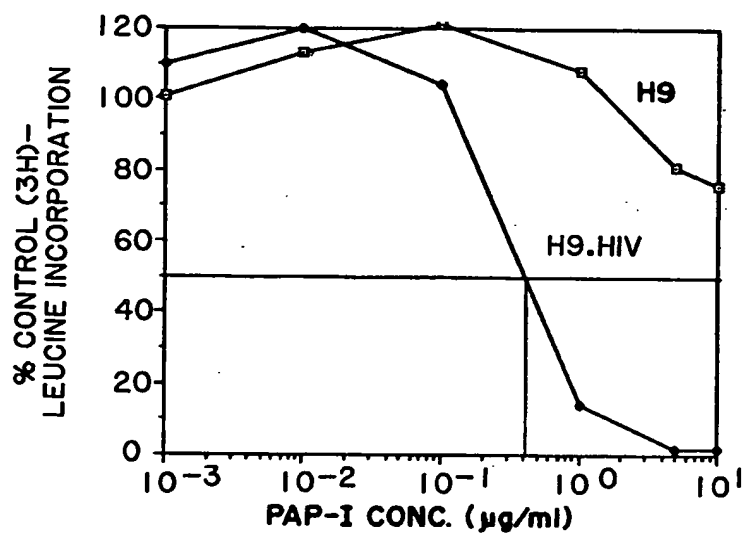


FIG. 18B



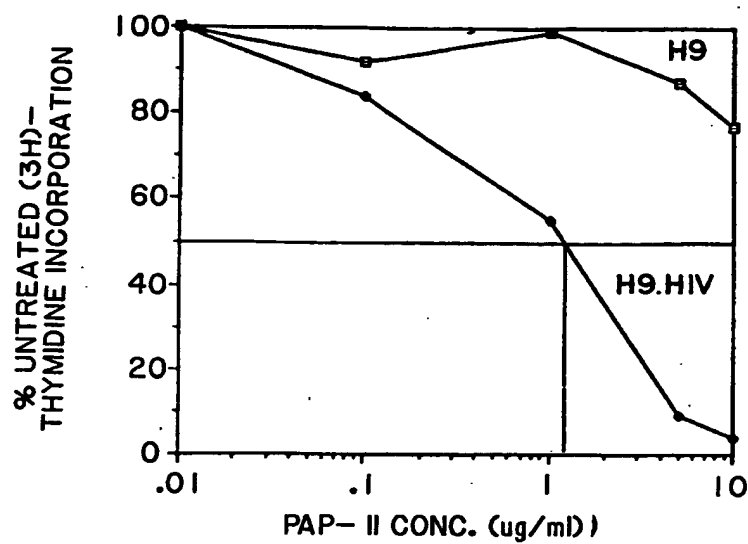


FIG. 19

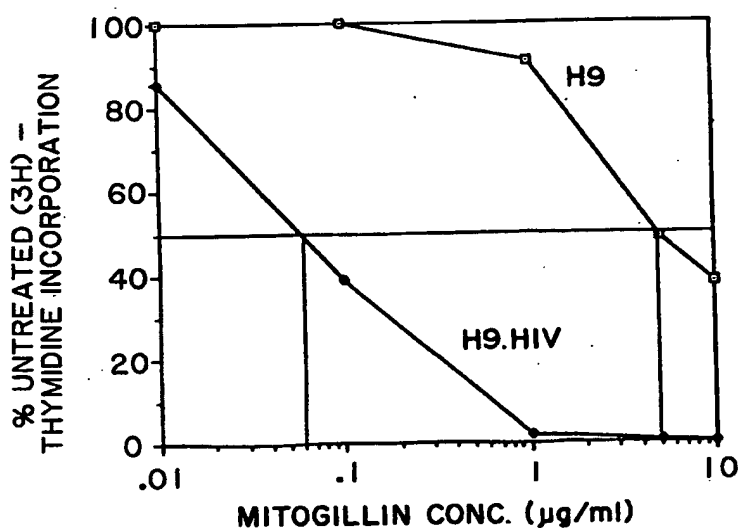


FIG. 20

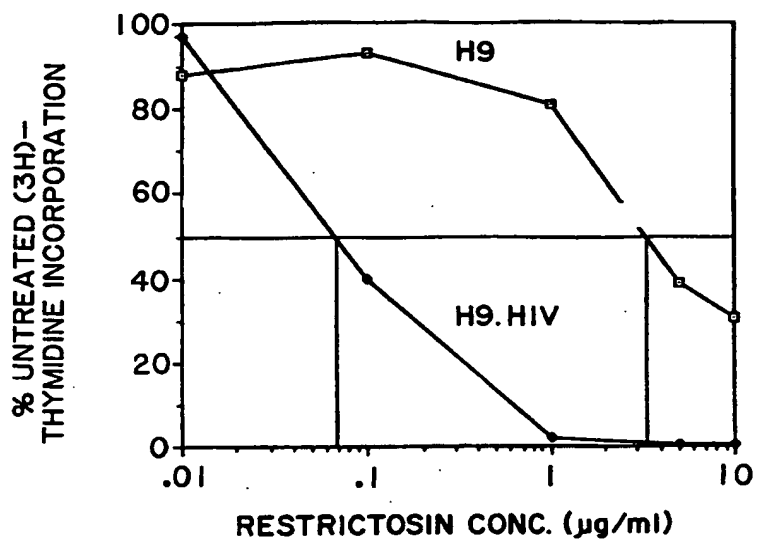


FIG. 21

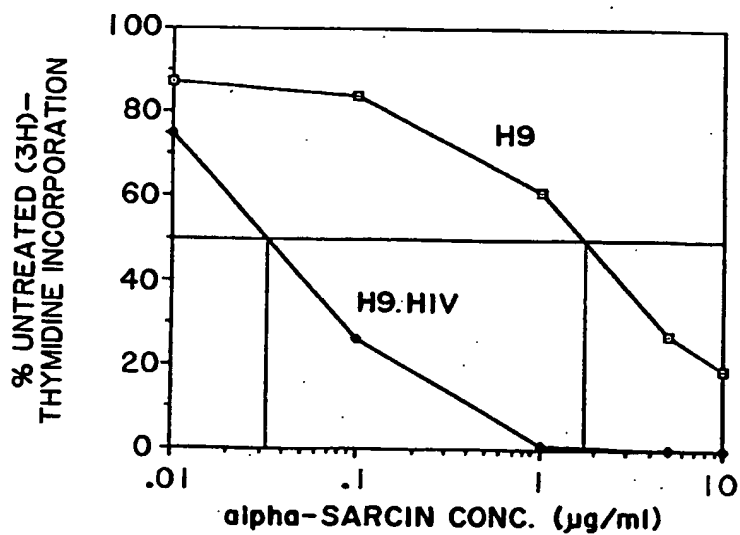


FIG. 22

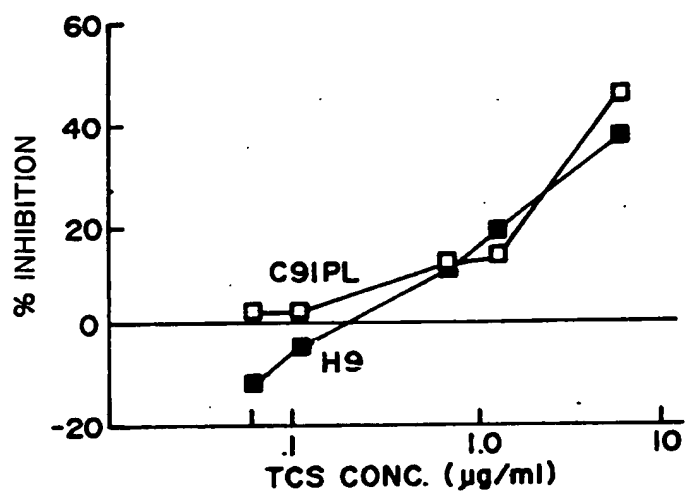


FIG. 23A

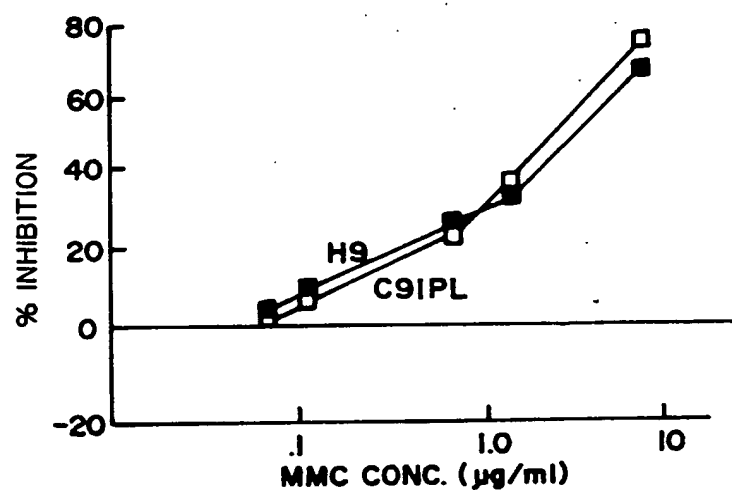


FIG. 23B

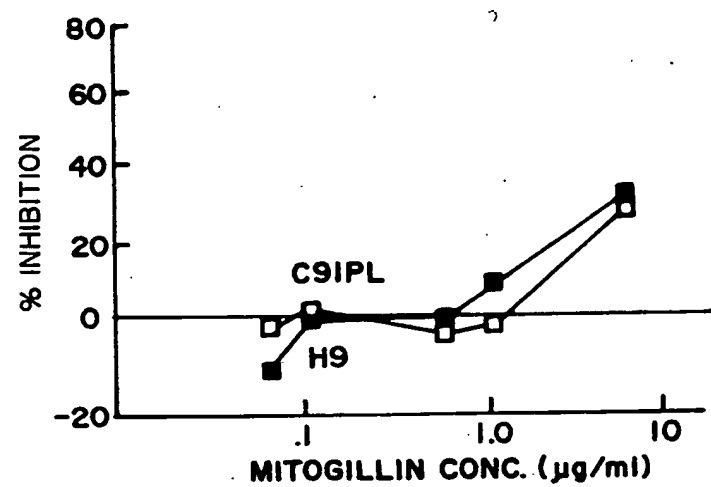


FIG. 23C

# METHOD OF SELECTIVELY INHIBITING HIV

The present invention is a continuation-in-part of copending U.S. patent application for "Method of Inhibiting HIV", Ser. No. 056,558, filed May 29, 1987.

## FIELD OF THE INVENTION

The present invention relates to a method of inhibiting the expression of HIV proteins in human T cells and monocyte/macrophages, as a method of treating HIV infection in humans.

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## BACKGROUND

Human Immunodeficiency Virus (HIV) is a retrovirus which is the etiological agent for acquired immune deficiency syndrome (AIDS) and a spectrum of related disorders (Coffin). The virus is transmitted by parenteral inoculation and/or intimate sexual contact. It is estimated that approximately 2 million people in the United States are infected with HIV at present, and current projections are that a majority of those now infected will develop AIDS or a significant clinical HIV-related disease within a 7-10 year follow-up period (Barnes).

HIV is tropic and cytopathic for cells which express the cell surface differentiation antigen CD4 (T4, leu3). The viral tropism is believed to result from interactions between CD4 and the envelope glycoprotein of HIV. These interactions appear to be involved in the process by which HIV infects susceptible cells, and also underlie the mechanism by which HIV induces cell fusion in T cells (Lifson, 1986a, 1986b; Dalglish; Klatzman, 1984a, 1984b; Maddon; McDougal, 1985a, 1985b; Sodroski). The cell fusion process, which can lead to cell death, may, in turn, contribute to the progressive depletion of CD4 cells which characterizes AIDS, and which may be a factor contributing to HIV-induced immunocompromise and its secondary consequences, opportunistic infections and neoplasms (Fahey).

The host cell range for the Human Immunodeficiency Virus (HIV) includes, in addition to CD4+ T cells, cells of the mononuclear phagocytic lineage, including peripheral blood monocytes, tissue macrophages, (Steicher), Langerhans cells of the skin, and dendritic reticulum cells (Armstrong) within the lymph nodes. Mononuclear phagocytes may be a primary target cell for HIV infection within the central nervous system (Koenig; Gartner, 1986b). Cells of the macrophage lineage are likely to represent a major viral reservoir in vivo, and either alone or through their interactions with T cells, may contribute to the development and pathogenesis of AIDS and related clinical diseases (Crowe). Experiments conducted in support of the present invention suggest that a large percentage of monocyte/macrophages derived from HIV-infected individuals are capable of expressing HIV antigens, indicating widespread infection of the macrophage precursors. There is also evidence that macrophages expressing the HIV surface antigen may interact and fuse with CD4+ T cells, leading to destruction of the crucial T cells (Crowe).

Intensive efforts to develop therapies which can prevent or block the development of serious clinical symptoms in HIV-infected individuals are under way. For the most part, these efforts have focused on the use of nucleoside analogue drugs which inhibit the viral reverse transcriptase enzyme (Yarochan, 1986, 1987; Broder, 1985, 1987). These drugs would be expected to selectively inhibit new viral infection of cells, such as T cells and monocyte/macrophages, since reverse transcriptase is required for early viral infection. However, once viral infection is established in a cell, and viral replication is then carried out using host cell enzymes, the reverse transcriptase inhibitors would be expected to have limited inhibitory effect on viral replication and expression of viral antigens on the host cell surface. In vitro studies have demonstrated HIV replication even in the continued presence of nucleoside analogues in prolonged culture. Although indications of some beneficial clinical effects have been observed, the early clinical testing results have provided little evidence that these drugs will be effective against later-stage progression of HIV infection to serious clinical diseases.

#### SUMMARY OF THE INVENTION

It is one general object of the invention to provide a method of selectively inhibiting HIV replication and proliferation of HIV infected human T cells and mononuclear phagocytic lineage cells.

A related object of the invention is to provide a method of inhibiting HIV antigen expression in HIV-infected cells.

Another related object is to provide a method of selectively inhibiting HIV antigen expression in mononuclear phagocytic lineage cells.

It is yet another object of the invention to provide a method of treating HIV infection in humans.

The discovery that viral expression and cell proliferation in HIV-infected T cells and monocyte/macrophages can be selectively inhibited by two plant proteins—trichosanthin (TCS), and momorcharin (MMC)—was described in the above, earlier filed patent application for "Method of Inhibiting HIV". An important aspect of the discovery was the finding that viral inhibition in HIV-infected cells could be achieved at concentrations of TCS or MMC which were substantially non-toxic to uninfected cells. This selective effect

was evidenced by a marked decrease in viral antigen associated with HIV-infected cells, and in measurable reverse transcriptase activity, several days after exposure to TCS or MMC, without a significant decrease in expression of non-viral protein in non-infected cells. Another aspect of the selective inhibitory effect of TCS and MMC which was observed was a substantial loss of cell viability in HIV-infected cells, at an MMC or TCS concentration which did not significantly reduce the viability of non-infected cells. Typically, these selective inhibition effects were seen at TCS or MMC concentrations between about 0.01–3.0 ug/ml.

As discussed in the earlier filed application, the selective inhibitory effect of TCS and MMC may be related to the proposed ribosome inhibitory activity of these proteins. MMC is a potent inhibitor of protein synthesis in a cell-free system (Barbieri, 1982); and it has been speculated, from the observed amino acid homology between TCS and the A chain of ricin, that TCS may have ribosome inactivating properties similar to ricin and various single-chain plant proteins or glycoproteins which have N-glycosidase activity, and which have been reported to inactivate ribosomes by glycoside cleavage at one or more selected sites in ribosomal RNA (rRNA).

It has now been discovered that a broad range of single-chain proteins with ribosome inactivating activity, including gelonin, various species of pokeweed anti-viral proteins, alpha-sarcin, restrictocin, and mitogillin, also produce selective inhibition of viral expression in HIV-infected cells. It has also been discovered that the selective inhibitory effects are virus specific, as evidenced by lack of selective inhibition in T cells infected with HTLV-I virus, a related, but distinct human retrovirus.

The selective inhibitory effect in HIV-infected cells may be demonstrated by (a) selective inhibition of viral antigen expression in HIV-infected mononuclear phagocytic lineage cells; (b) selective inhibition of cellular proliferation, as measured against protein and DNA synthesis levels in treated, noninfected T cells; and (c) selective loss of T cell viability. These inhibitory effects have been observed for several representative single-chain plant and fungal ribosome inactivating proteins (scRIPs).

It has further been found that the selective inhibitory effects in HIV-infected cells can be achieved by continuous cell exposure to the scRIP, at a selected scRIP concentration, or by short-term or pulsed exposure, e.g., 30–120 minutes, of HIV-infected cells to the scRIP. In the pulse-exposure approach, the concentration of scRIP and time of exposure can be selected to produce nearly complete inhibition of HIV-infected T cells, without appreciable inhibition in noninfected cells.

In one aspect the method of the invention includes exposing HIV-infected T cells and mononuclear phagocytic lineage cells to a single-chain ribosome inactivating protein (scRIP), at a concentration and duration of exposure which are effective to produce a substantial reduction in viral antigen expression in the cells. Since the inhibitory effect of the scRIPs in mononuclear phagocytic lineage cells is selective for viral antigen expression, the scRIP dose/exposure time can be selected to produce inhibition of viral protein expression in HIV-infected cells, with substantially less inhibition of cellular protein synthesis in the HIV-infected cells. Alternatively, since the scRIPs are selectively inhibitory of cellular proliferation in HIV-infected T cells,

the scRIP dose/exposure time can be selected to produce inhibition of cell proliferation in HIV-infected T cells, as measured, for example, by selective loss of cell viability in the HIV-infected cells, or by inhibition of thymidine uptake in the cells, without significantly inhibiting these parameters in uninfected T cells. Typically, the concentration of anti-HIV protein to which the cells are exposed is between about 0.01 and 1 ug/ml.

The ability of the scRIPs to inhibit HIV antigen expression in infected cells is exploited, according to another aspect of the invention, for treating HIV infection in humans. The method includes administering a single-chain ribosome inactivating protein to the subject, at a dose which is effective to produce a measurable decrease in at least one of the following indications of HIV infection:

- (a) HIV antigen levels associated with HIV-infected T cells or mononuclear phagocytic lineage cells;
- (b) HIV antigen levels in the bloodstream;
- (c) the reverse transcriptase activity associated with HIV-infected T cells or mononuclear phagocytic lineage cells;
- (d) the ratio of viability of HIV-infected to uninfected T cells; and
- (e) the ratio of a selected HIV antigen to a selected cellular antigen in HIV-infected mononuclear phagocytic lineage cells.

The decrease in indication(s) is preferably measurable within 1-5 days after protein administration. The method may additionally include alternatively measuring the decrease in at least one of the indications, and repeating the protein administration, until the measured indication of HIV infection shows no further decrease.

Preferred scRIPs include plant-derived and fungal-derived scRIPs, such as trichosanthin, momorcharin, pokeweed antiviral protein, alpha-sarcin, mitogillin, and restrictocin.

These and other objects and features of the invention will become more fully apparent when the following detailed description of the invention is read in conjunction with the accompanying drawings, wherein:

#### BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1A-1C show chromatography elution profiles obtained in the isolation of trichosanthin;

FIGS. 2A-2C show chromatography elution profiles obtained in the isolation of alpha and beta momorcharin;

FIGS. 3A-3H are cytofluorographic plots (linear scale) of T cells reacted first with control (nonspecific) antibody (frames at the left) or human anti-HIV antibody (frames at the right) followed by fluorescein-labeled anti-human antibody for uninfected T cells (3A,B); HIV-infected T cells (3C,D); and uninfected or HIV-infected T cells 16 days after exposure to 10 ug/ml TCS (3E,F) or MMC (3G,H).

FIG. 4 shows percent inhibition of p24 HIV antigen expression in HIV-infected T cells as a function of concentration of TCS (open and closed squares) and MMC (triangles);

FIG. 5 shows percent inhibition of p24 HIV antigen expression in HIV-infected T cells as a function of concentration of PAP-I (open squares), PAP-II (triangles), and PAPS (closed squares);

FIG. 6 shows percent inhibition of p24 HIV antigen expression in HIV-infected T cells as a function of concentration of mitogillin (open squares), restrictocin (triangles), and alpha-sarcin (closed squares);

FIG. 7 shows the decrease in HIV replication in infected cells treated with TCS and MMC, as evidenced by decreased levels of particle associated reverse transcriptase (RT) detectable in the culture supernatant harvested 5 days after addition of TCS (solid diamonds) or MMC (open squares), where the solid squares indicate lack of RT activity in uninfected cells;

FIGS. 8A and 8B are cytofluorographs of permeabilized uninfected (8A) and HIV-infected (8B) monocyte/macrophages after labeling with mouse anti-p24 antibody and fluorescein-tagged anti-mouse antibody;

FIG. 9 shows the increase in percent macrophages containing expressed viral p24 antigen as detectable by permeabilized fluorescence analysis, as a function of time after in vitro infection with HIV, in normal macrophages derived from each of two donors (open circles and triangles), and the change in cell viability which occurs in the cells during the same period (closed circles and triangles);

FIGS. 10A-10D are cytofluorographs of uninfected (10A) and HIV-infected (10B) monocyte/macrophages, and infected macrophages treated with 5 ug/ml of TCS (10C) or 5 ug/ml MMC (10D) four days prior to assaying for the presence of p24 viral antigen, as in FIG. 8;

FIG. 11 is a plot showing the increase in HIV p24 antigen expression during a 10-day period after monocyte/macrophages isolated from an HIV-infected patient are first placed in culture (open squares), and the inhibition of p24 expression when the cells are exposed to 0.3 ug/ml TCS when the cells are first placed in culture (closed circles) or when TCS (0.3 ug/ml) is added after 4 days in culture (closed squares), when a large proportion of the cultured cells are already expressing p24 antigen;

FIG. 12 is a plot showing the change in percent of HIV-infected monocyte/macrophage cells with detectable levels of HIV p24 antigen (as detectable by permeabilized fluorescence analysis), as measured at 0, 24, and 96 hours after exposure of the infected cultures to 5 ug/ml of TCS (closed triangles), MMC (open circles), or no drug treatment (solid circles);

FIG. 13 is a plot showing the change in percent of HIV-infected monocyte/macrophage cells with detectable levels of HIV p24 antigen, measured as in FIG. 8 four days after exposure to 0, 0.5 or 5 ug/ml of TCS (open triangles) or MMC (open circles);

FIG. 14 shows the percent of p24 antigen expression, with respect to untreated cells (100%), four days after exposure to 0, 0.005, 0.05, 0.5, and 5 ug/ml MMC (triangles);

FIG. 15 shows the extent of inhibition of cellular proliferation, as measured by percent <sup>3</sup>H-thymidine incorporation into cellular DNA in HIV-infected T cells (H9.HIV) and uninfected cells (H9), expressed as a function of TCS concentration;

FIGS. 16A and 16B show the extent of inhibition of cellular proliferation, as measured by percent <sup>3</sup>H-thymidine incorporation into cellular DNA in HIV-infected T cells (H9.HIV) and uninfected cells (H9), as a function of time of exposure to TCS (16A) or MMC (16B) at 2 ug/ml;

FIGS. 17A and 17B are plots showing the absolute viable cell counts for identically seeded cultures of HIV infected (H9.HIV) and uninfected (H9) T cells cultured in the presence of varying concentrations of TCS for 2 days (FIG. 17A) or 5 days (FIG. 17B), and FIG. 17C shows the percentage of viable cells present after 2 days



in identically seeded cultures treated with varying concentrations of TCS;

FIGS. 18A and 18B show percent inhibition of cellular proliferation of HIV-infected (H9.HIV) and non-infected (H9) T cells with exposure to increasing concentrations of PAP-I, as evidenced by inhibition of <sup>3</sup>H-thymidine incorporation into cellular DNA (18A), and inhibition of <sup>3</sup>H-leucine incorporation into cellular protein (18B);

FIG. 19 shows the extent of inhibition of cellular proliferation, as measured by percent <sup>3</sup>H-thymidine incorporation into cellular DNA in HIV-infected T cells (H9.HIV) and uninfected cells (H9), as a function of PAP-II concentration;

FIG. 20 shows the extent of inhibition of cellular proliferation, as measured by percent <sup>3</sup>H-thymidine incorporation into cellular DNA in HIV-infected T cells (H9.HIV) and uninfected cells (H9), as a function of mitogillin concentration;

FIG. 21 shows the extent of inhibition of cellular proliferation, as measured by percent <sup>3</sup>H-thymidine incorporation into cellular DNA in HIV-infected T cells (H9.HIV) and uninfected cells (H9), as a function of restrictocin concentration;

FIG. 22 shows the extent of inhibition of cellular proliferation, as measured by percent <sup>3</sup>H-thymidine incorporation into cellular DNA in HIV-infected T cells (H9.HIV) and uninfected cells (H9), as a function of alpha-sarcin concentration; and

FIGS. 23A-23C shows percent inhibition of cellular proliferation, as measured by percent <sup>3</sup>H-thymidine incorporation into cellular DNA in HTLV-I-infected T cells (open squares) and uninfected T cells (closed squares), measured at increasing concentrations of TCS (23A), MMC (23B), and mitogillin (23C).

## DETAILED DESCRIPTION OF THE INVENTION

### I. Definitions

The terms below have the following meanings herein, unless indicated otherwise:

1. "HIV" means a CD4+-dependent (T4, leu3-dependent) human immunodeficiency retrovirus as exemplified by HIV-1 and known variants thereof.
2. "Mononuclear phagocytic lineage cells" means CD4+ mononuclear phagocytes, including CD4+ peripheral blood monocytes, peritoneal macrophages, Langerhans cells of the skin, dendritic reticulum cells of the lymph nodes, pulmonary macrophages, Kupfer cells of the liver, and monocyte/macrophage cells.
3. "Monocyte/macrophage cells" are mononuclear phagocyte lineage cells present in peripheral blood which are precursors of blood macrophages. The cells are obtained from peripheral blood or human spleen biopsies and cultured in cell culture.
4. "T cells" means either transformed T lymphoid cell lines susceptible to HIV infection, or HIV-susceptible T cells derived from primary, peripheral blood mononucleate cell preparations.
5. "HIV-infected cells" means HIV-infected T cells and/or mononuclear phagocytic lineage cells.
6. "Uninfected cells" means T cells and/or mononuclear phagocytic lineage cells which are not infected with HIV.
7. "Single-chain ribosome inactivating proteins (scRIP)" refer to single-chain proteins or peptides capable of inhibiting protein synthesis in a cell-free

protein synthesizing system, by site-specific enzymatic inactivation of eukaryotic ribosomal RNA as described in Section IV below.

### II. Selective Inhibition of HIV Antigen Expression

This section examines the parameters of selective inhibition of HIV antigen expression in HIV-infected human cells by scRIPs. The human cells which are specifically described are T cells, whose destruction *in vivo* is associated with loss of immunological function in advanced clinical HIV infection; and monocyte/macrophages, a type of mononuclear phagocytic lineage cell, as defined above, which are likely provide a reservoir of the virus in the infected individual, and may, when expressing HIV proteins, be capable of fusing with and destroying T cells. The monocyte/macrophages may also play a role in the spread of the infection, particularly as within the nervous system (Koenig).

#### A. T Lymphocyte Cells

Normal human T lymphocytes can be prepared from peripheral blood or from lymphoid solid tissue by standard procedures (Foung). The cells include a fraction of CD4+ cells which may be further isolated, if desired, by affinity methods which are specific for the CD4+ surface antigen. In the usual case, the mixture of CD4+ and nonCD4+ cells are employed. The cells are maintained in culture over a several-day to several-week period by activation with a known lymphocyte mitogen, such as PHA, and in standard cell media, such as RPMI-1640 medium supplemented with fetal calf serum and interleukin-2. The cultured T lymphocytes may be infected with HIV *in vitro*, e.g., using an HIV isolate derived from an AIDS patient.

In addition, continuous T cell lines, typically derived from patients with lymphoid malignancies, may also be employed. Such cells may be maintained in standard cell culture media, such as RPMI 1640 supplemented with heat-inactivated fetal calf serum.

One characteristic of HIV-infected T cells is the appearance of HIV envelope proteins, particularly the major envelope proteins gp120 and gp41, on the surface of the infected cells. As indicated above, gp120 appears to play a critical role in the recognition of CD4+ T lymphocytes, cytes, and the subsequent destruction of these lymphocytes. gp41 may also participate in HIV envelope mediated cell fusion (Kennedy). Therefore, the ability of the scRIPs herein to inhibit expression of these viral antigens may be an important indicator of the ability of the protein to inhibit virally mediated processes leading to T cell destruction and to arrest the loss of immunological function seen in HIV-related diseases, such as AIDS. The demonstrated antiviral properties are not, however, limited to action via this mechanism.

The effects of TCS and alpha-MMC on the expression of HIV antigens (including gp120 and gp41) on the surface of infected T cells were examined, as detailed in Example 3. The effect of continuous TCS and MMC exposure on HIV antigen expression are shown in FIG. 3. HIV infected T cells (H9 cell line) were treated with 10 ug/ml of TCS or alpha-MMC for 16 days, then tested for HIV antigen expression by indirect immunofluorescence analysis. Briefly, human serum from an HIV-positive individual was incubated with test cells, followed by washing and detection of bound specific IgG with a fluoresceinated goat anti-human IgG reagent.

gent. The results of the quantitative flow cytometry analysis are shown in FIG. 3, and discussed in Example 3. The results show that both TCS and MMC reduced the level of HIV antigen expression in treated, HIV-infected T cells to background levels.

Another HIV antigen which can be used as an indicator of HIV antigen expression in HIV-infected T cells is the HIV core protein antigen p24. This antigen, which is localized predominantly within the infected cells, can be measured readily by treating the cells with a membrane-permeabilizing agent, such as Triton X-100™, to facilitate entry of anti-p24 mouse monoclonal into the cells, then treating the cells with a fluorescent-tagged anti-mouse IgG antibody. These methods are detailed in Example 4. Alternatively, viral replication may be quantified by determining the amount of HIV p24 antigen present in either cell-free supernatants of infected cultures or in lysates of infected cells, through use of a sandwich type antigen-capture immunoassay. Both approaches provide a measure of viral antigen expression or production, i.e., the amount of HIV antigen produced by the infected cells and detectable by the particular assay means used.

The effect of low concentrations of several scRIPs on p24 expression in HIV-infected T cells was examined, according to procedures detailed in Example 4. Briefly, HIV-infected and noninfected T cells were cultured with the selected scRIP, at continuous-exposure concentrations of between 0.01 and 5 ug/ml, for 10 days, then assayed for p24 antigen as above. FIG. 4 shows the percent inhibition of p24 produced by the indicated TCS and MMC concentrations. The data indicate substantially complete inhibition of viral antigen at scRIP continuous exposure concentrations of 1 ug/ml.

FIG. 5 shows the effect of three pokeweed antiviral protein preparations on p24 expression in HIV-infected T cells. The results are qualitatively similar to those produced by TCS and MMC, although slightly higher scRIP concentrations were required to produce complete inhibition of p24 expression.

In FIG. 6, the inhibitory effect of three fungal scRIPs on p24 expression in HIV-infected cells is shown. As seen, the lowest concentrations tested produced complete or nearly complete inhibition of p24 expression in infected T cells. The apparently greater specific activity of the fungal scRIPs may be due in part to their lower molecular weights (Section I), and thus greater molar concentration at the indicated ug/ml concentration levels.

It has been confirmed that the inhibition of HIV antigen expression discussed above represents an inhibition of viral replication, as indicated by a loss of reverse transcriptase (RT) activity in the infected cells. This feature is seen in FIG. 7, which plots the reverse transcriptase activity in HIV-infected T cells, as a function of TCS or MMC concentration. The fall in RT activity correlates well with inhibition in HIV surface antigen expression in infected cells (FIG. 3).

As will be seen in Section III below, the 0.1-1 ug/ml concentrations of scRIP required to produce substantially complete inhibition of HIV antigen expression in infected T cells produces little if any inhibition of cellular proliferation in noninfected T cells. This selectivity effect in T cells can thus be exploited to inhibit HIV infection in infected T cells, with little toxic effect on uninfected cells.

## B. Mononuclear Phagocytic Lineage Cells

Since mononuclear phagocytic lineage cells (MPLC) represent another group of HIV infectable cells, and probably provide a significant reservoir of the virus in humans, the inhibitory effect of scRIPs against HIV infection in this cell group is also of interest. The cell type which has been examined is the monocyte/macrophage, a mononuclear macrophage-lineage cell present in the peripheral blood, and a precursor of tissue macrophages. These cells, which will be referred to herein for simplicity as "monocytes", can be isolated from peripheral blood or from human spleen biopsies by known procedures (Crowe). The cells are preferably cultured in a novel in vitro culture system which employs Teflon® coated culture vessels, as described in Example 6. Upon in vitro culture, peripheral blood derived monocytes undergo differentiation, acquiring some characteristics of tissue macrophages. For isolation of monocytes, peripheral blood mononuclear cells are allowed to attach to a glass dish, permitting separation of the monocytes from non-adherent lymphocytes. The separated monocytes are then cultured as a suspension on Teflon®-coated dishes. Although the cells do not proliferate, they can be maintained in a viable state in culture for up to four months or more without significant loss of cell viability.

The cells can be infected in vitro with an HIV isolate, as described in Example 6, or obtained in infected form from an HIV-infected individual, by similar methods. HIV antigen expression in monocyte/macrophages can be readily followed by changes in p24 antigen, as outlined above. FIGS. 8A and 8B show cytofluorographs of HIV-infected monocytes reacted with control (non-specific) or mouse anti-p24 antibodies, respectively, measured 10 days after HIV infection in vitro. About 60% of the cells have detectable (above background) fluorescence levels, indicating HIV infection.

One important feature of the monocyte cell culture system, for studying HIV infection and inhibitors, is that the cells can be maintained in an actively infected state in culture for extended periods of several weeks or more. This feature is seen in FIG. 9, which plots monocyte viability (closed symbols) and p24 antigen levels (open symbols) as a function of days after infection in vitro with HIV, where the circles and triangles represent two different monocyte donors. Experimental details are given in Example 6. As seen, viral antigen increases steadily to a maximum of about 50-60% infected cells, then plateaus after about two weeks, while cell viability remains substantially unchanged over the test period.

The inhibition of viral antigen expression in HIV-infected monocytes, when exposed to TCS or MMC, is illustrated in FIG. 9. Here, monocytes were exposed to 5 ug/ml TCS or MMC ten days after HIV infection, and four days later, the cells were assayed by flow cytometry. FIGS. 10A and 10B show the cytofluorographs of untreated cells analyzed after reaction with control (non-specific) and anti-p24 antibody, similar to FIGS. 8A and 8B, respectively. The cytofluorographs of TCS- and MMC-treated cells are shown in FIGS. 10C and 10D, respectively. The level of p24 seen in the scRIP-treated cells (FIGS. 10C, 10D) is close to background (FIG. 10A), indicating substantially complete inhibition of viral antigen expression four days after cell exposure to anti-viral proteins. Example 7A gives details of the method.

The time course of loss of p24 antigen in infected monocytes after exposure to TCS or MMC is seen in FIG. 12. Each time point represents percent cells with an above-background antigen-specific fluorescence, determined as above by flow cytometry. Untreated cells (solid circles) show little change in antigen levels, whereas both TCS (triangles) and MMC (open circles) treated cells show a severalfold reduction in viral antigens 1 day after, and substantially complete loss of p24 antigen 4 days after exposure to TCS or MMC. Details of the study are given in Example 7B.

The data in FIG. 13, which plot the inhibition in p24 expression as a function of TCS or MMC, show that both TCS and MMC produce nearly complete inhibition of p24 expression at a concentration of 0.5 ug/ml. In FIG. 14, the percent inhibition of p24 with 3 hour pulse exposure to MMC, at concentrations as low as 0.005 ug/ml, is seen. The data show nearly complete inhibition at 5 mg/ml concentration.

According to another aspect of the invention, it has been found that the inhibitory effect of scRIP on HIV-infected monocytes is selective for viral proteins, at least when the infected cells are given a pulse exposure to a low dose of the scRIP. Specifically, it has been found that HIV-infected monocytes, when exposed to a low-concentration pulse dose of scRIP, show a marked reduction in measurable p24 (FIG. 14), but no significant reduction in measurable cellular surface antigens, as exemplified by the surface antigen HLA-DR. Details of the study are provided in Example 7D.

The presence of HIV in macrophages isolated from AIDS patients, and the inhibition of HIV antigen expression in monocytes infected in vivo and isolated from a infected donor was also examined. Several monocyte preparations from both peripheral blood and spleen cells from AIDS patients were tested for p24 antigen expression. The macrophage cultures were established essentially as described in Example 4, except that the donors were HIV seropositive, and no exogenous virus was added to accomplish in vitro infection. The source of HIV in the culture was that present from natural, in vivo infection of the cell donor. Monocytes tested immediately after isolation contained only a small percentage of HIV positive cells, as evidenced by the presence of p24 antigen. The percentage of cells expressing p24 increased gradually over a 3 to 4 day period in culture, as indicated for a culture of spleen monocytes (open squares) in FIG. 11. In five different monocyte preparations derived from HIV-seropositive individuals which have been examined, the cultured cells expressed between about 10%-40% p24 after 3-4 days in culture. The results indicate that a high proportion of monocytes present in HIV seropositive individuals are infected with HIV. Apparently only a small percentage of those infected cells express HIV antigens unless cultured for short periods in vitro. The possibility that the increase in number of cells expressing p24 is caused by the spread of the virus among the cultured cells is unlikely, given the relatively slow rate of p24 expression in newly-infected cultured monocytes (FIG. 7).

The inhibitory effect of TCS on p24 expression in the in vivo infected cells was examined both at the initiation of the culture, when the percentage of p24 expressing cells was quite low and, in a separate culture, 5 days after initiation of culture, at which time p24 expression was observed in about 45% of the cells. FIG. 11 shows the results of TCS treatment (0.3 ug/ml) of  $5 \times 10^5$  monocytes cultured over a ten day period. TCS added

at the initiation of the culture completely prevented HIV antigen expression over the 10-day test period. When added to the 5-day monocyte culture, TCS reduced the percentage of cells expressing p24 from about 45% to 2% within three days, and further reduced the percent of antigen-expressing cells to background level within 5 days. It is clear from the data that TCS can block HIV antigen expression in monocytes derived from an infected individual, either before or after antigen expression occurs in culture.

The methods and findings which are discussed herein can be summarized as follows:

1. Initial findings, presented in the copending patent application for "Method of Inhibiting HIV" demonstrated that low concentrations of TCS and alpha- and beta-MMC effectively inhibit HIV antigen expression in HIV-infected T cells and mononuclear macrophage lineage cells. Low concentrations (e.g., less than about 1 ug/ml) of the same compounds gave only slight inhibition of protein synthesis in non-infected cells.

2. The above selective inhibitory effects on viral expression in infected T cells and monocyte/macrophages have now been demonstrated for several additional single-chain ribosome inactivating proteins, including scRIPs from both plant and fungal sources.

3. In addition to the selective inhibitory effects seen in HIV-infected T cells and monocyte/macrophages (as evidenced by the lack of protein inhibition produced in non-infected cells at low scRIP concentrations), scRIP are capable, at low concentrations and/or low exposure times, of selectively inhibiting viral protein synthesis in HIV-infected monocyte/macrophages.

### III. Selective Inhibition of T Cell Proliferation

Studies presented in the earlier-filed copending patent application showed that TCS, gave a marked selective reduction in cell proliferation and viability in HIV-infected cells. This section examines the parameters of selective inhibition of cellular proliferation in HIV-infected cells. The selective inhibitory effect of several representative scRIPs on cell proliferation, at low dose levels, has now been established. In addition, it has been found that essentially complete selectivity can be achieved by pulse dosing. It is also shown that the selective effects are relatively specific for HIV infection, since no appreciable selective inhibitory effects are observed in T cells infected with a related, but distinct human retrovirus, HTLV-I.

Inhibition of cellular proliferation was determined by following the inhibition of thymidine uptake in treated cells, and in some cases, this approach was supplemented with data on inhibition of amino acid uptake and/or reduction in cell viability. Details of the cellular inhibition studies are given in Examples 8 and 9.

To determine effective inhibitory dose of scRIP, the noninfected and HIV-infected cells were exposed to increasing quantities of the selected scRIP, typically at concentrations between 0.01 and 5 ug/ml, and over an exposure period of three days. The cells were then pulsed with radiolabeled thymidine for 12 hours and radiolabel incorporated into cellular DNA was measured, as an index of cellular proliferation. FIG. 15 shows the levels of thymidine incorporation measured at 0.01, 0.1, 1, 5, and 10 ug/ml TCS, where H9 and H9.HIV indicate noninfected and HIV-infected T cells, respectively. As seen, 50 percent inhibition of infected cells is achieved at about 0.2 ug/ml, whereas the same degree of inhibition in noninfected cells requires nearly

10 ug/ml TCS. The selectivity index, defined as the ratio of the two concentrations, is thus about 50 for TCS. Similar results (not shown) have been obtained for MMC.

As seen from FIG. 15, the maximum selectivity effect (greatest difference between the two curves) was observed at 1 ug/ml TCS. According to one aspect of the invention, it has been discovered that the selectivity of inhibition between infected and noninfected cells can be enhanced substantially by exposing the cells to a pulse dose of the selected scRIP, as distinguished from continuous exposure. This effect is seen in FIGS. 16A and 16B, which show the inhibition of thymidine uptake as a function of exposure time to 2 ug/ml TCS (16A) or MMC (16B). As shown, neither scRIP tested gave appreciable inhibition of thymidine uptake by noninfected cells after exposure to the compounds for up to two hours. By contrast, a 2 hour exposure, with no subsequent exposure, was sufficient to produce a nearly complete inhibition of cellular proliferation in the infected cells, when measured three days later.

FIGS. 17A-17C show the effect on cell viability of increasing concentrations of TCS, as reported in the earlier-filed patent application. Viable cell numbers, 2 and 5 days following exposure to TCS at the concentration indicated, are plotted in FIGS. 17A and 17B, respectively, and percent viable cells, in FIG. 17C, where cell viability was determined by vital dye exclusion (trypan blue).

The cell viability data in the figures shows that infected cells are more susceptible to killing, particularly at higher concentrations of TCS, with a more pronounced effect observed at 2 days than at 5 days. This likely reflects the fact that less than one hundred percent of the cells within the "infected" population were actually productively infected. Cells which are not productively infected appear to be less susceptible to TCS and MMC; the apparent relatively lesser effect of drug exposure on cell counts at day 5 may reflect preferential outgrowth of individual cells within the "infected" population which were not themselves productively infected. Comparison of total viable cell numbers with percentage viability suggests that TCS, in a dose-dependent fashion, can exert both cytotoxic (cell killing) and cytostatic (cell growth inhibiting) effects, with preferential effects at a given peptide concentration on HIV infected cells relative to uninfected cells. Similar results were obtained with alpha and beta-MMC.

FIGS. 18A and 18B show the degree of cellular inhibition of H9 and H9.HIV cells with exposure to increasing concentrations of PAP-I. As seen in FIG. 18A, the 50 percent inhibitory levels and selectivity index for thymidine incorporation are very similar to those seen for TCS. FIG. 18B demonstrates that the inhibition of amino acid (leucine) incorporation into treated H9 and H9.HIV cells closely follows the inhibition of thymidine uptake.

FIGS. 19-22 show selective inhibition curves for H9 and H9.HIV cells treated with PAP-II (FIG. 19), mitogillin (FIG. 20), restrictocin (FIG. 21), and alpha-sarcin (FIG. 22). In all cases, the selectivity index was between about 50-70. Consistent with the viral antigen inhibition data discussed in Section III above, the data here indicate that the three fungal proteins tested are inhibitory at concentrations somewhat lower than observed with plant scRIPs.

The inhibitory effect of scRIPs on T cells infected with HTLV-I was also examined. HTLV-I (human

T-cell leukemia/lymphoma virus) has several features in common with HIV, including a tropism for OKT4-/leu3+ lymphocytes, cytes, a Mg<sup>2+</sup>-dependent high molecular weight reverse transcriptase, similar size and nature of some structural proteins, and limited nucleic acid homology (Sarnagadharan).

T cells infected with HTLV-I were exposed to increasing concentrations of TCS, MMC, or mitogillin, according to methods described in Example 10. The inhibition of thymidine incorporation, as a function of scRIP concentration, for both noninfected (H9) and infected (C9/PL) T cells is shown in FIGS. 23A-23C, for the three proteins tested. No selective inhibition was observed with any of the tested compounds. Specifically, HTLV-I infected cells were no more susceptible to inhibition by the compounds than noninfected H9 cells.

The methods and findings which are discussed herein can be summarized as follows:

1. Initial findings, presented in the copending patent application for "Method of Inhibiting HIV" demonstrated that TCS and MMC can selectively inhibit cellular proliferation in HIV-infected T cells, as evidenced both by inhibition of thymidine uptake and reduced cellular viability.

2. The selective inhibitory effects on cellular proliferation in infected T cells have now been demonstrated for several additional A-chain like plant and fungal proteins. All of the tested compounds show a selectivity index of between about 50-70 when tested under assay conditions involving continuous exposure of HIV-infected or uninfected cells to the compounds.

3. By pulse dosing infected T cells with the scRIPs, essentially complete selectivity (i.e., no measurable inhibitory effects on noninfected cells) can be achieved.

4. The selectivity effects are specific for HIV, as evidenced by the lack of any selectivity in T cells infected with a related, but different human retrovirus, HTLV-1.

#### IV. Single-Chain Ribosome-Inactivating Proteins

The scRIP proteins which are useful in the present invention can be classed generally into one of four groups, depending on source of the protein (plant, fungal, or bacterial) and subunit structure of the naturally occurring protein (single-chain or two-subunit).

The first group includes single-chain inhibitors of protein synthesis which are produced by a large number of plants. Examples of proteins in this group are TCS (Law, Kuo-Fen, Gu, Xuejun, Wang, Kezhen), MMC (Falasca, Spreafico, Lin, 1970, 1978, Barbieri, 1979, 1980), the pokeweed antiviral proteins (Barbieri, 1982, Irvin, 1975, 1980, 1983), gelonin (Stirpe, 1980), dianthin 30 and dianthin 32 (Stirpe, 1981), croton II (Conde), curcin II (Conde), wheat germ inhibitor (Roberts), and several other protein inhibitors obtained from grains (Coleman, Gasperi-Campani).

A second group of scRIPs are obtained from fungal sources and occur naturally in single-chain peptide form. This group is exemplified by alpha-sarcin (Rodriguez, Olson, 1965a, 1965b), restrictocin (Rodriguez), mitogillin (Rodriguez), enomycin (Conde) and phenomycin (Conde).

Some plants produce cytotoxins which are composed of two dissimilar subunits or peptide chains—an enzymatically active A chain having ribosome inactivating activity specific for the ribosomal RNA of the large ribosome subunit, and a B chain which functions to bind

the toxin to cell-surface receptors (Olsnes, 1982). The best-known cytotoxins of this type are ricin, abrin, and modeccin, all of which are similar in structure and mechanism of action. The subunits in these proteins are linked through a disulfide bond, which can be broken under reducing conditions (Olsnes, 1982), allowing purification of the individual subunits. The A subunits of the cytotoxins, i.e., the ribosome inactivating subunits, form the third class of scRIPs, in accordance with the present invention.

The fourth group of scRIPs are single-chain bacterial protein or peptide cytotoxins, such as the cytotoxin from *Shigella dysenteriae*, and related Shiga-like toxins (Calderwood). These toxins are composed of one A chain-like subunit, similar to the A chains of the plant cytotoxins described above and having the identical mode of action (Endo, 1987), and multiple B subunits having cell binding functions. These A subunits form the fourth group of scRIPs.

Several lines of evidence indicate that the scRIPs in all four groups share common structural features and have a common mechanism of action. The A chains of ricin, modeccin, abrin, all show regions of amino acid sequence homology (Olsnes, 1987), and a number of single-chain plant RIPs, including pokeweed antiviral proteins (Irvin, 1975), wheat germ inactivating (Roberts), MMC (Barbieri), gelonin (Stirpe), dianthins (Stirpe), and TCS (Maraganore), resemble the ricin A chain in primary peptide structure. The Shiga-like bacterial toxins also show sequence homology with ricin A (Calderwood). The primary structures of restrictocin, mitogillin, and alpharsarin all show a high degree of sequence homology (Rodriguez).

The scRIPs defined herein also share a common mechanism of ribosome inactivating activity, which involves site specific enzymatic action on the large ribosomal RNA (rRNA) of the 60S ribosomal subunit (Olsnes). In several of the scRIPs which have been investigated, including the A chains of ricin, abrin, and modeccin, the single-chain plant inhibitors pokeweed antiviral proteins, crotin II, curcin II, the fungal proteins mitogillin, alpha-sarcin, restrictocin, eomycin, phenomycin, and Shiga-like bacterial toxins, catalytic inactivation of the 60S subunit of eukaryotic ribosomes has been reported (Conde). In the case of many plant inhibitors, including ricin, abrin, modeccin, and pokeweed antiviral protein, the mechanism of ribosomal inactivation involves an N-glycosidase activity which removes the adenine from a specific adenosine nucleotide subunit. For the fungal inhibitors which have been investigated to date, including alpha-sarcin, mitogillin, and restrictocin, the inactivation event involves phosphodiester cleavage at a specific site close to the cleavage site of the N-glycosidase inhibitors (Endo, 1982, 1987).

Another feature common to single-chain ribosome inactivating proteins which has been reported is enhanced inhibition of protein synthesis in certain virus-infected cell systems. For example, it has been observed that gelonin, dianthin-32, MCI (*M. charantia* inhibitor) and PAP-S all produce a greater inhibition of protein synthesis in HEp-2 cells infected with either herpes simplex virus (HSV-1) or with poliovirus I than in noninfected cells (Foa-Tomasi). The levels of inhibitor protein required to achieve selective inhibitory effects were typically between 10-100 ug/ml, and the degree of inhibition seen was typically less than about 50% (FIG. 3 of Foa-Tomasi).

More recently, it has been shown that several single-chain inhibitors, including alpha-sarcin, mitogillin, restrictocin, PAP, and the abrin A chain, selectively inhibit protein synthesis in HeLa cells infected by the picornavirus encephalomyocarditis virus (EMC). Selective inhibition was seen at relatively low inactivating concentrations of alpha-sarcin and abrin A chain. Selective inhibition of HeLa cells infected with adenovirus and BHK cells infected with Semliki Forest virus was also observed. Further studies indicated that binding of EMC virus or poliovirus to surface receptors on a virus-infectable host cell may increase the permeability of the cell to inactivating protein (Fernandez-Puentes, 1980, 1983).

Although viral infection may enhance the protein inhibitory effects of single-chain ribosome inactivating proteins on infected cells, this effect appears to depend strongly on the specific virus and infected cell type involved. For example, in the above-mentioned study on virus-infected HeLa cells, infection with EMC showed a substantially greater selective inhibition of protein synthesis than in the same cells infected with adenovirus. In the same reference, BHK cells infected with Semliki Forest virus showed, at most, about a 40% difference in protein inhibition between infected and non-infected cells. And, as indicated above, in HEp-2 cells infected with either poliovirus or HSV-1, selective protein inhibition effects were only observed at high inactivating concentrations, and the maximum difference in degree of inhibition, between infected and non-infected cells, was about 50%.

From the findings presented in Section III above, it is seen that T cells infected with different retroviruses show dramatic differences in their response to scRIPs. In particular, HTLV-I infection of human T cells results in no detectable selective inhibitory action by scRIPs. By contrast, in T cells infected with HIV, a distinct but closely related retrovirus, differences in inhibitory effect of up to 80-90% are observed at low inactivating concentrations of between about 0.1-1 ug/ml concentration. Further, when the cells are exposed to a pulsed dose, differences in selective inhibitory effects up to 100% are observed.

It was earlier shown (copending patent application for "Method of Inhibiting HIV") that low doses of TCS and MMC produced a marked selective inhibition of viral antigen production in HIV-infected T cells and monocyte/macrophages, and a selective reduction in cell viability in infected T cells. This discovery has now been extended to a total of eight scRIPs which are representative of single-chain plant inhibitors and fungal inhibitors. In addition, it has now been shown that the scRIP treatment of HIV-infected T cells and/or monocyte/macrophages produces several important effects which have not been observed heretofore. These are:

(a) Substantially complete inhibition of HIV antigen expression and viral replication in HIV-infected T cells and monocyte/macrophages, at scRIP concentrations which are substantially nontoxic to uninfected cells;

(b) Selective loss of viral antigen with respect to cellular antigen in virus-infected monocyte/macrophages; and

(c) Substantially the same degree of selective inhibition produced by a variety of different scRIPs which were tested. In particular, as noted in Section III, all of the plant and fungal compounds tested gave a selectivity index for inhibition of cellular proliferation of be-

tween about 50-70. This contrasts with prior art reports where the selectivity effects varied widely among ribosome inactivating proteins which were tested. This result, taken together with the representative nature of the eight scRIP compounds which were tested, indicates that the marked selectivity effects presented herein can be reasonably generalized to include compounds of the four groups of scRIPs defined above.

#### V. Treating HIV Infection in Humans

One link in the etiology of AIDS appears to be the destruction of CD4+ T lymphocytes, and there is in vitro and in vivo evidence to suggest that at least one mechanism of cell destruction involves fusion of infected cells to form large multi-nucleate cells. The inventors and their coworkers have previously studied the relationship between expression of the CD4 antigen and infectability by HIV (Lifson, 1986a-c). The studies confirmed earlier reports that HIV infection of T lymphocytes requires the CD4 antigen, suggesting that the infection process requires interactions between the CD4 antigen and one or more envelope proteins of HIV (Dalglish; Klatzman, 1984a, 1984b; McDougal, 1985a, 1985b, 1986; Maddon; Sodroski). The previous study by the inventors also showed that infected T cells can fuse with both infected and non-infected CD4+ T cells in vitro to produce large multi-nucleate syncytia, and that cell fusion can be blocked with addition of anti-CD4 antibodies. This indicates that cell fusion, like HIV infection, requires interactions between viral antigens on the surface of infected cells and the T lymphocyte CD4 antigen (in either infected or non-infected cells). The result may also explain how a large portion of CD4+ T cells can be destroyed in vivo, even though only a relatively small number of isolated CD4+ T cells from an HIV-infected individual show evidence of HIV infection. According to this mechanism, infected T lymphocytes would "recruit" healthy T cells for cell fusion and destruction.

Using T lymphocytes selected for high CD4+ antigen expression, the inventors have further shown in previous studies that (a) the infectability of the T lymphocytes with HIV increases substantially with increased surface concentration of the antigen, and (b) syncytia formation due to cell fusion is much more rapid in the high CD4+ cells. The results support earlier findings on the importance of the CD4+ antigen in HIV infection and subsequent cell-fusion events.

There is also evidence that monocyte/macrophages may be involved the etiology of HIV infection. It has been reported that cells of the monocyte/macrophage lineage can be infected with HIV in vitro (Crowe, Gartner, 1986a, 1986b; Koenig; Ho; Chayt; Armstrong; Steicher), and monocytes have been implicated in the spread of HIV into the central nervous system (Koenig). The studies reported in Section III on monocytes prepared from HIV-infected patients indicate that a large percentage of blood and splenic macrophages may harbor HIV infection, even though viral antigens may be actively expressed in vivo in a relatively small percentage of the cells. In addition to providing a possible reservoir of HIV in the body, macrophages may also be directly involved in the destruction of T lymphocytes by cell fusion. Recent studies by the inventors and their coworkers show that HIV-infected monocytes are capable of fusing readily with non-infected CD4+ lymphocytes, forming giant multi-nucleate syncytia

(Crowe). There is also evidence of macrophage involvement in HIV infection of the CNS (Koenig).

It can be appreciated from the foregoing how the selective viral inhibition effects produced by scRIPs, in accordance with the present invention, can be applied to treating HIV infection in humans. The ability of scRIPs to selectively inhibit HIV replication, as evidenced by substantially complete inhibition of viral antigen expression and reverse transcriptase activity, would reduce the level of infection by reducing the production of new virus capable of infecting new cells. Further, inhibition of viral replication may help to eliminate the virus "reservoir" which may be provided by the monocyte/macrophages and other cells. In this regard, it is noted that at least one scRIP—TCS—appears to be able to cross the blood/brain barrier (Hwang) and thus should be effective against the spread of HIV infection to the CNS.

Secondly, evidence discussed above suggests that the fusion of CD4+ T cells with infected T cells or monocyte/macrophages requires the presence of HIV antigens on the surface of the infected cells. One striking effect of the anti-HIV proteins is the ability to inhibit and substantially eliminate expression of viral antigens in infected cells. Substantial reduction in the expression of viral proteins would be expected to be associated with a marked reduction in the cytopathogenic consequences of infection.

Another therapeutic advantage of HIV treatment with scRIPs, in accordance with the invention, is the selective inhibition of HIV-infected T cells. Since infected T cells have the potential to fuse with noninfected T cells, to cause accelerated depletion of the total T cell population, selective destruction of the infected cells should limit the extent of "secondary" T cell destruction.

The anti-HIV proteins can also be expected to inhibit or prevent other events related to the loss of immunological competence in HIV infected individuals, through general suppression of virus levels and inhibition of viral protein synthesis in infected cells.

The dose level of an scRIP which will be therapeutically effective will depend on a variety of factors which can be readily monitored in a treated human subject. Selective inhibition of HIV replication and cellular proliferation in T cells has been observed at scRIP doses as low as 0.01 ug/ml, particularly for the fungal-derived scRIPs tested. For some scRIPs, or for more acute stages of infection, dose levels up to 1 ug/ml or more may be required. However, even with long term exposure to the scRIP, concentrations in the 1-2 ug/ml range are not severely inhibitory in noninfected cells (Section IV). Concentrations of scRIP in the bloodstream of between 0.01 and 1 ug/ml, assuming an approximately 3.50 liter plasma volume, can be achieved by administering a total scRIP dose of between about 0.03 to 3 mg of the scRIP. In the case of TCS, this dose compares with the 5-12.5 mg dose of TCS which is used for inducing abortion in humans, and this dosage level is not generally associated with serious side effects in humans (Kuo-Fen; Hwang).

In the treatment method of the invention, an scRIP is administered to an HIV-infected no line break subject, at a dose which is effective to produce a measurable decrease in at least one of the following indications of HIV infection:

(a) HIV antigen levels associated with HIV-infected T cells or mononuclear phagocytic lineage cells;

(b) HIV antigen levels in the bloodstream;  
 (c) the reverse transcriptase activity associated with HIV-infected T cells or mononuclear phagocytic lineage cells;

(d) the ratio of viability of HIV-infected to uninfected T cells; and

(e) the ratio of a selected HIV antigen to a selected cellular antigen in HIV-infected mononuclear phagocytic lineage cells.

Preferably, the indication of HIV inhibition which is monitored is plasma antigen levels, e.g., serum or plasma p24 levels which can be readily followed using an ELISA procedure. Preferably, the decrease in indication(s) is measurable within 1-5 days after the compound administration. The method may further include alternatively measuring the decrease in at least one of the indications, and repeating the scRIP administering, until the measured indication of HIV infection shows no further decrease.

When a series of doses are administered, e.g., over a several-week period, the patient should be monitored for allergic response to the anti-HIV protein. If a serious response does develop to the first-administered scRIP, e.g., TCS, a second scRIP, e.g., MMC, can be administered to minimize immunological reaction and neutralization of the protein. Preliminary animal data developed by one of the inventors suggest that the two proteins are substantially immunologically non-cross reactive. However, since many patients who would be receiving the treatment are seriously immune compromised, immune response to the proteins may be a relatively minor side effect.

The protein may be administered parenterally in one of a variety of delivery forms, including solution form, liposome-encapsulated form, and attached to a carrier, such as an anti-T cell, anti-macrophage, or anti-HIV antibody, for targeting the protein to HIV-infectable or infected cells. Methods for preparing and storing peptide drug formulations of various types, and for administering the formulation by intravenous, intramuscular, subcutaneous, mucosal membrane, and inhalation routes are known in the pharmaceutical industry.

The following examples illustrate various methods and uses of the present invention, and typical anti-HIV effects observed in the screening method of the invention. The examples are intended to illustrate, but in no way limit, the scope of the invention.

#### Materials

##### A. Reagents

[3]H-thymidine and [3]H-leucine were obtained from New England Nuclear; and fluorescein isothiocyanate (FITC) conjugated goat-anti-human IgG reagent, from Zymed (Burlingame, Calif.); Anti-p24 monoclonal antibody was provided by Dr. J. Carlson, UC Medical Center, Davis, Calif.

##### B. HIV isolates

The DV strain of HIV was used for all experiments involving in vitro infection of monocyte/macrophages from noninfected donors. This is a lowpassage isolate obtained from the peripheral blood of a heterosexual man with Kaposi's sarcoma (Crowe). Several liters of the high titre stock of the virus were grown in the VB T lymphoma cell line (Lifson, et al, 1986 a-c), and aliquots were stored at -70° C. until used. Stock cultures of HIV-DV contained about  $5 \times 10^5$  infectious units/ml, where an infectious unit is defined as the amount of

infectious virus required to produce characteristic cytopathic effects (CPE) by day 5 of culture, when inoculated onto  $5 \times 10^5$  VB indicator cells. Stock cultures contained about  $89 \times 10^3$  cpm of reverse transcriptase activity, as measured by published methods (Hoffman). Other experiments, as specified, utilized monocyte/macrophages obtained from HIV-infected donors. In these experiments, the HIV isolate employed was the autologous isolate in the particular patient. For T cell studies, the HXB-2 isolate of HIV was used.

##### C. T Cells

H9 T lymphocyte cells were derived from the H9 cell line (Popovic) VB cells, a T lymphoblastoid cell line were obtained from Dr. S. Smith (Stanford University). Fluorescence activated cell sorting using a fluorescent-labeled anti-CD4 antibody, was used to obtain a strongly CD4-expressing subline of VB. The cells were maintained in RPMI-1640 supplemented with 10% (v/v) heat inactivated fetal calf serum.

##### D. Monocyte/Macrophages

Human macrophage cultures were established from peripheral blood mononuclear cells obtained from leukapheresis preparations of human blood, and buffy coats from normal blood donors, according to established methods (Crowe). Briefly, peripheral blood mononuclear cells were isolated by density centrifugation over Ficollhypaque, and allowed to attach to glass petri dishes in RPMI 1640 medium supplemented with 20% fetal calf serum at 37° C. for 1 hour. After washing (to remove contaminating, non-adherent lymphocytes), the monocytes were recovered from the petri dishes by placing on ice for 10 minutes in 5 uM EDTA-PBS, 5%FCS medium and scraping with a rubber policeman. The recovered monocyte preparation was then centrifuged, resuspended in RPMI 1640 medium with 10% pooled male HIV-negative human serum (complete medium) and placed into Teflon® culture vessels at  $2 \times 10^6$  cells per ml. Cell viability decreased over the first five days in culture to a stable density of approximately  $5 \times 10^5$  cells per ml. Long term cultures were maintained at this density for up to four months. Medium was routinely changed every 7 days.

##### E. Ribosome Inactivating Proteins

###### 1. Trichosanthin

Trichosanthin (TCS) is a plant protein which is obtained from the *Trichosanthes kirilowii* root tuber. The protein, which is also known as alpha-trichosanthin (Law) and Radix trichosanthis (Kuo-Fen), is a basic, single-chain protein consisting of between about 224 (Gu) to 234 (Xuejun) amino acid residues, and having a molecular weight of about 24,000 daltons. One preferred purification method for obtaining TCS in purified form is outlined in Example 1 below. This method involves three chromatographic separations which are shown in FIG. 1. The protein sequence of TCS has been completed (Gu; Wang), and a molecular model has been derived from cytofluorographic X-ray analysis (Kerhan).

###### 2. Momorcharin

Momorcharin (MMC) is a basic glycoprotein obtained from the seeds of the bitter melon plant *Momordica charantia*. The protein appears to have two related forms which have been designated alpha and beta



momorcharin. Alpha-momorcharin has a reported molecular weight of between about 31,000 to 32,000 daltons and a neutral sugar content of about 1.6%. Beta-momorcharin has a reported molecular weight of about 29,000 daltons and a neutral sugar content of about 1.3 % (Chan). Both forms of momorcharin are effective in inhibiting HIV antigen expression in HIV-infected T lymphocytes and monocyte/macrophages, according to the invention. Momorcharin is defined herein to include both alpha and beta momorcharin, as well as active portions of these proteins which are effective in inhibiting HIV antigen expression in HIV-infected blood cells. The plant glycoprotein can be isolated to homogeneity by fractionating an acetone extract from the seeds of *M. charantia* on CM Sepharose CL-6B, and Sephadex G100, according to published methods (Yeung, 1985), and as detailed in Example 2 below. FIG. 2 shows the three chromatographic separations involved in the MMC. The proteins are homogeneous on fractionation by HPLC on a TSK250 gel permeation column, SDS gel electrophoresis and immunoelectrophoresis.

Momorcharin appears to be related and perhaps even identical to one or more *M. charantia* inhibitors ("MCI") having molecular weights in the 30,000 to 32,000 dalton range, and possessing ribosome-inhibitory activity in cell-free systems. Such inhibitors which have been described in the literature are a *Momordica charantia* inhibitor, having molecular weights of 31,000 daltons (Falasca) or 30,000 daltons (Spreafico); "agglutinin", having a molecular weight of about 32,000 daltons (Lin 1970, 1978); and possibly one or more of the four subunits (molecular weights 30,500, 29,000, 28,500 and 27,000 daltons) in a hemagglutinating lectin obtained from the seeds of *M. charantia* (Barbieri, 1980).

It is noted that the MCI which has been most extensively studied as a ribosome inactivating protein was originally characterized as a 23,000 dalton protein, although a subsequent molecular weight determination yielded a molecular weight of about 31,000 daltons (Falasca).

### 3. Pokeweed Antiviral Protein-I (PAP-I)

PAP-I is a plant, single-chain protein of approximately 27,000 daltons molecular weight. The protein was supplied by Dr. James D. Irvin and was prepared from the pokeweed plant *Phytolacca americana*, according to published methods (Irvin, 1975). Briefly, *P. americana* leaves obtained from young plants were homogenized in distilled water, filtered, and the filtrate brought to 40% ammonium sulfate and centrifuged to remove precipitated material. The resulting supernatant was chromatographed by DEAE ion-exchange chromatography. A protein peak which contained all of the PAP protein inhibitory activity was further fractionated by phosphocellulose ion-exchange chromatography. The peak which eluted at 0.12M KCl and which contained the bulk of the protein inhibitory activity was used.

### 4. Pokeweed Antiviral Protein-II (PAP-II)

PAP-II is another single-chain ribosome-inhibitory protein obtained from *P. americana*. The protein is a single polypeptide chain of approximately 29,000 daltons molecular weight. The protein was supplied by Dr. James D. Irvin and was prepared by published methods (Irvin, 1980), involving a modification of the purification method for obtaining PAP-I. Briefly, the elution fractions from the final phosphocellulose col-

umn used in the PAP-I purification gave two major peaks of protein inhibitory activity. The first peak corresponds to PAP-I. The second peak was taken as PAP-II. Like PAP-I, the PAP-II protein strongly inhibits eukaryotic protein synthesis in a cell-free system, and also inhibits tobacco mosaic virus transmission. The two proteins are distinct, however, in their molecular weights, tryptic peptide maps, and immunological properties.

### 5. Pokeweed Antiviral Protein-S (PAP-S)

PAP-S is a third distinguishable single-chain ribosome-inhibitory protein obtained from *P. americana*. The protein is a single polypeptide chain of molecular weight about 30,000 daltons. The protein was supplied by Dr. James D. Irvin and was prepared by published methods (Barbieri). PAP-I, PAP-II, and/or PAP-S, are also referred to more generally herein as "pokeweed antiviral protein".

### 6. Alpha-Sarcin

Alpha-sarcin is a fungal, single-chain polypeptide of approximately 17,000 daltons molecular weight (Sacro). The protein was supplied by Dr. R. Amils, Universidad Autonoma de Madrid, Madrid, Spain and can be obtained from *Aspergillus giganteus*, according to published methods (Olson, 1963a, 1965b). Briefly, a fermentation of *A. giganteus* was filtered through a plate and frame press and the washed extract was adsorbed onto a column of carboxylic acid resin equilibrated to pH 7.0. Elution at 1.5M KCl gave two peaks with alpha-sarcin activity, and these were concentrated, combined, and treated with activated charcoal to remove some contaminating proteins. The crude material was further fractionated on a carboxylic acid ion-exchange resin, using a 0.4M to 0.9M phosphate buffer gradient. Alpha-sarcin eluted as a substantially pure protein.

### 7. Restrictocin

Restrictocin, like alpha-sarcin, is a fungal, single-chain polypeptide chain of approximately 17,000 daltons molecular weight. The protein was supplied by Dr. R. Amils and can be obtained from *Aspergillus restrictus* by published methods (Olson, 1963).

### 8. Mitogillin

Mitogillin is a related fungal protein which also has a molecular weight of about 17,000 daltons. The protein was supplied by Dr. R. Amils, and can be obtained from *Aspergillus restrictus* according to published methods (Olson, 1966).

## EXAMPLE 1

### Preparation of trichosanthin

All steps of the procedure described below were carried out at 4° C. Fresh root tubers of *T. kirilowii* were peeled, sliced into small pieces, and homogenized in a Waring blender in normal saline at pH 7 (0.7 l/kg). The homogenate was filtered through cheesecloth to remove debris. The extract was adjusted to pH 8 with 1N NaOH, stirred for 2 h, and then left to stand overnight. The suspension was centrifuged at 5,000 rpm for 15 min, and the resulting supernatant was adjusted to pH 8 with 1N NaOH. After standing for 1 h, cold acetone (0.8 ug/ml solution) was added. A heavy precipitate resulted and the mixture was stirred for 30 min. The precipitate (API) was collected by centrifugation at 5,000



rpm for 15 min. To the supernatant cold acetone (1.2 ug/ml supernatant) was added and after standing for 2 h, the sedimented material (AP2) was recovered by centrifugation. The two fractions AP1 and AP2 were suspended in a minimal volume of phosphate-buffered saline (PBS) and after extensive dialysis against the same buffer, the supernatant and the precipitate of each fraction were separated by centrifugation. The precipitate was then reextracted with PBS, centrifuged and the supernatant combined with the previous supernatant to form the subfraction S, while the precipitate after the reextraction formed the subfraction P. Thus, fraction AP1 gave two subfractions AP1S and AP1P. The yields of the various fractions are: 3.2 g AP1S; 3.8 g AP2S from 1 kg of sliced fresh tuber.

Fraction AP2S (1 g) was dissolved in about 10 ml of 0.05M phosphate buffer (pH 6.4) and applied to a column of CM-Sephacrose CL-6B (Pharmacia). The column had previously been equilibrated with 0.05M phosphate buffer, and initial elution was with the same buffer. After the third peak C3 (FIG. 1A) had been eluted, a linear gradient of 0-0.3M NaCl in the same buffer was applied, as seen in FIG. 1A. The protein peak designated C6 was collected, dialyzed against distilled water, and lyophilized to yield highly purified trichosanthin. The average yield and percentage recovery of trichosanthin from AP2S are about 180 mg (18%).

Highly purified trichosanthin was also obtained from fraction AP1S by an additional chromatographic step with DEAE-Sephacrose CL-6B column. AP1S (1 g) was dissolved in 14 ml distilled water. After dialysis against 0.02M  $\text{NH}_4\text{HCO}_3$  containing 0.1M NaCl (pH 8.0), it was applied to a column of DEAE-Sephacrose CL-6B (1.5x32 cm) previously equilibrated with the same buffer. Column chromatography was carried out at room temperature at a flow rate of 70 ml/h and the eluate was collected in 10 ml fractions. The column was washed with 100 ml of the starting buffer (0.02M  $\text{NH}_4\text{HCO}_3$ ) containing 0.1M NaCl and then stepwise elution was performed with 0.2M NaCl and 0.5M NaCl in the same bicarbonate buffer. Elution was monitored by absorbance at 280 nm. The eluate was pooled into four fractions D1-D4 (identified in FIG. 1B), dialyzed and lyophilized. The average yield and percentage recovery of the trichosanthin-enriched fraction D1 from AP1S is about 185 mg (18.5%).

The trichosanthin-enriched fraction D1 was further purified on a CM-Sephacrose CL-6B column by a similar procedure as described above for AP2S. The protein peak designated P6 (FIG. 1A) was collected and stored at  $-70^\circ\text{C}$ , or dialyzed against distilled water, and lyophilized to yield highly purified trichosanthin. The average yield and percentage recovery of trichosanthin from D1 (1 g) is about 445 mg (44.5%).

The combined (from AP2S and D1) yield and percentage recovery of trichosanthin from 1 kg of sliced fresh tuber of *T. kirilowii* are about 900 mg (0.09%)

#### EXAMPLE 2

##### Preparation of alpha and beta momorcharin

Decorticated dried ripe seeds (100 g) of *Momordica charantia* were homogenized in 0.9% saline (about 4 ml per 1 g) with a Waring Blender and filtered through cheesecloth. The pH of the filtrate was adjusted to 4.0 with 2N HCl before centrifugation at 12,000 rpm for 20 min. The supernatant (crude extract) was then subjected to acetone fractionation at  $4^\circ\text{C}$ . To the crude

extract, 0.8 v/v of cold acetone ( $-20^\circ\text{C}$ ) was slowly added with constant stirring and the mixture was kept at  $4^\circ\text{C}$  for 1 h before centrifuged at 5,000 rpm for 15 min to remove the precipitate (API). Cold acetone ( $-20^\circ\text{C}$ ) was then added to the supernatant to achieve a final concentration of 2.0 v/v. After standing at  $4^\circ\text{C}$  for 1 h, the mixture was centrifuged at 5,000 rpm for 15 min to recover the precipitate (APII) which was resuspended in and dialyzed against 0.05M phosphate buffer (pH 6.4) and applied to a column of CM-Sephacrose CL-6B (Pharmacia) equilibrated with the same buffer. Initial elution was with the same buffer. After the third peak had been eluted, a linear gradient of 0-0.2M NaCl in the same buffer was applied, as seen in FIG. 2A, which shows the elution profile from the column. The protein peaks designated  $\text{C}_{5b}$  and  $\text{C}_{6a}$  were collected, and stored at  $-70^\circ\text{C}$ , or dialyzed against distilled water, and lyophilized. The average yields and percentage recoveries from APII are:  $\text{C}_{5b}$  (136 mg, 17%) and  $\text{C}_{6a}$  (76 mg, 9.5%).

The protein fractions  $\text{C}_{5b}$  (50 mg) and  $\text{C}_{6a}$  (50 mg) were separately dissolved in 2.5 ml phosphate-buffered saline (pH 7.2) and the undissolved precipitates removed by centrifugation before being applied onto a Sephadex G-100 (fine) (Pharmacia) column equilibrated and eluted with the same buffer. The major protein peaks designated  $\text{C}_3\text{-G}_1$  (peak  $\text{G}_1$  in FIG. 2B) and  $\text{C}_6\text{-G}_1$  (peak  $\text{G}_1$  in FIG. 2C) were collected, and stored at  $-70^\circ\text{C}$ , or dialyzed against distilled water and lyophilized to yield alpha-momorcharin ( $\text{C}_3\text{-G}_1$ ) and beta-momorcharin ( $\text{C}_6\text{-G}_1$ ), respectively. The average yields and percentage recoveries from  $\text{C}_{5b}$  and  $\text{C}_{6a}$  are: alpha-momorcharin (35 mg, 72%) and beta-momorcharin (32 mg, 64%).

Yields and percentage recoveries of alpha-momorcharin and beta-momorcharin from 1 kg of decorticated dried seeds of *Momordica charantia* are about 800 mg (0.08%) and 400 mg (0.04%), respectively.

#### EXAMPLE 3

##### Effect of TCS and MMC on HIV Antigen Expression in HIV-Infected T Cells

To evaluate the effect of TCS and alpha MMC on viral antigen expression (including expression of the pathogenetically significant envelope antigens gp 120 and gp 41), HIV-infected H9 cells were seeded at  $0.5 \times 10^6/\text{ml}$  and cultured in RPMI-1640 supplemented with 10% heat inactivated fetal calf serum, in the presence of TCS or alpha-MMC at concentrations ranging from 0.3 to 10 ug/ml. Culture medium was replaced every 3-5 days with fresh medium containing additional TCS or alpha-MMC to maintain the specified concentration. At various time points, cells were assayed for expression of HIV antigens by indirect immunofluorescence analysis. Briefly, for each assay,  $1 \times 10^6$  cells were washed with phosphate buffered saline containing 1% HI-FCS (staining buffer). Cells were then incubated with a 1:50 dilution of previously characterized HIV antibody positive or negative serum in staining buffer at  $4^\circ\text{C}$  for 45 minutes. After washing, bound specific antibody was detected with a fluorescein conjugated goat anti-human IgG reagent. Quantitative flow cytometric analysis was performed on an Ortho Cytofluorograf 50 H. FIGS. 3A to 3H show the results of flow cytometric analysis performed at day 16 of culture. FIGS. 3A and 3B demonstrate the lack of reactivity of the HIV antibody negative (3A) and antibody positive

(3B) patient serum with uninfected H9 cells. FIGS. 3C and 3D show specific reactivity of the antibody positive serum with HIV infected cells (3D) while HIV antibody negative serum does not react (3C). The antibody negative serum does not react with HIV infected cells treated with 10  $\mu$ g/ml of TCS (3E) or  $\alpha$ -MMC (3G). The antibody positive serum shows only background levels of reactivity (approximately 2% or fewer cells showing above threshold fluorescence) with the TCS treated (3F) and  $\alpha$ -MMC treated (3H) HIV infected cells, indicating the virtual absence of viral antigen expression by the treated cells. This dramatic phenomenon is noted most strikingly upon comparing FIG. 3D with FIGS. 3F and 3H.

#### EXAMPLE 4

##### Effect of scRIP on HIV p24 Antigen Expression in HIV-Infected T cells

VB cells were treated with the indicated concentrations of scRIP concomitant with inoculation with infectious, cell-free HIV virus. Cells were seeded at  $0.5 \times 10^6$ /ml and cultured in RPMI-1640 supplemented with 10% heat inactivated fetal calf serum, in the continued presence of a selected concentration of TCS,  $\alpha$ -MMC, PAP-I, PAP-II, PAP-S, mitogillin, restrictocin or  $\alpha$ -sarcin. The scRIP concentrations are shown along the ordinate in FIGS. 4-6.

Cells were cultured for 3-5 days, and observed for development of characteristic HIV-induced cytopathology (Lifson, 1986a). Cell-free supernatants were harvested and tested using a commercially available ELISA format HIV antigen assay to assess viral replication, by measuring levels of HIV p24 core protein.

FIG. 4 shows the level of p24 produced by infected T cells, expressed relative to those produced by untreated infected cells, as a function of increasing concentrations of TCS (open and closed squares) and  $\alpha$ -MMC (triangles). Essentially complete inhibition of viral antigen expression was achieved at an scRIP concentration of between 0.5 and 1  $\mu$ g/ml for both MMC and TCS.

FIG. 5 shows the level of p24 produced by infected T cells, expressed as in FIG. 4 as a function of increasing concentrations of PAP-I (open squares), PAP-II (triangles) and PAP-S (closed squares). The results are similar to those seen in FIG. 4, although somewhat higher concentrations of PAP scRIP compounds were required to effect the same level of antigen inhibition.

FIG. 6 shows the level of p24 produced by infected T cells, expressed as in FIGS. 4 and 5 as a function of increasing concentrations of mitogillin (open squares), restrictocin (triangles), and  $\alpha$ -sarcin (closed squares). For all three scRIP compounds, substantially complete p24 inhibition was achieved at 0.5  $\mu$ g/ml concentration.

#### EXAMPLE 5

##### Effect of TCS and MMC Treatment on HIV Replication in Infected T cells

HIV replication was also assessed by measuring particle associated reverse transcriptase activity in cell-free supernatants of cultures. Infected and noninfected cells were seeded and exposed to several selected concentrations of TCS or  $\alpha$ -MMC as above. At 2 and 5 days after exposure to the drug, the cell suspensions were collected and pelleted by centrifugation at  $500 \times g$  for 10 minutes. The supernatant was then centrifuged at

45,000 g for one hour and reverse transcriptase (RT) activity was measured as described (Hoffman).

The results obtained for supernatants harvested on day 5 are plotted, as a function of concentration of the scRIP added, in FIG. 7. Uninfected cells (solid circles) showed no detectable RT activity. Both TCS (solid diamonds) and MMC (open squares) treatment resulted in significant, concentration dependent decreases in the amount of viral replication in the cultures, as assessed by measuring particle associated RT activity in cell free culture supernatants. At high concentrations of TCS virtually no RT activity was detected, particularly when one allows for the small amount of virus produced during the first several hours of treatment before the anti-HIV agents have had a chance to exert their effects.

#### EXAMPLE 6

##### Infection of Monocyte/Macrophages by HIV

##### A. Percent cells infected

Monocyte/macrophages were isolated and cultured as above. To infect the macrophage cells with HIV, the macrophages were pelleted by centrifugation after 5-15 days in culture, then resuspended in PBS with 2  $\mu$ g/ml of polybrene, followed by incubation at 37° C. for 30 minutes. After washing and centrifugation, the pelleted cells were resuspended in HIV-DV, at a concentration of  $5 \times 10^5$  macrophages and  $5 \times 10^5$  infectious units per ml. Cells were left in contact with the virus overnight at 37° C., then unbound virus was washed away, and the cells were resuspended at  $5 \times 10^5$  cells per ml in complete medium.

The frequency of HIV infected macrophages was determined by cytofluorographic, single-cell, indirect immunofluorescence, as described (Crowe), and as outlined in Example 4 above. FIG. 8 shows plots of cell counts as a function of fluorescence level for HIV-infected cells labeled with control (MOPC-21) antibody (A) or anti-p24 antibody (B). The data indicate that about 60% of the cells in the infected macrophage are reactive with anti-p24 antibody ten days after in vitro infection. A level of about 40-60% infected cells was routinely achieved with cells from other donors.

##### B. Long-term cell viability

To determine whether HIV-infected macrophages continue to survive long-term in culture, the cells were examined for p24 antigen expression over a several week period. The time course of p24 expression in infected macrophage cells from 2 different donors (open circles and triangles) is plotted in FIG. 9. As seen, p24 antigen expression increases rapidly over the first two weeks following infection (time zero), then continues at a relatively high level for at least about 4 weeks. Viability of infected cells from the two donors (closed circles and triangles in FIG. 7) was also examined, using trypan blue exclusion. The infected cells show almost no loss of viability over a four-week period in culture. No cytopathic effects were observed in the infected cells over the test period.

## EXAMPLE 7

Effect of TCS and MMC on HIV p24 Antigen Expression in HIV-Infected Monocyte/Macrophages  
A. Inhibition of p24 HIV Antigen Expression

Cultured macrophages prepared as above were carried in culture for 7 days, then exposed to TCS or MMC, at a final concentration of 5 ug/ml. Four days after addition of the drug, the cells were examined for p24 expression, as above, by indirect immunofluorescence with anti-p24 antibody. FIG. 10 shows the cytofluorographic profile of (A) control (uninfected) cells, (B), infected cells which were not exposed to drug, (C) infected, TCS-treated cells, and (D) infected, MMC-infected cells. As seen, four days of treatment with either drug reduces p24 expression in infected cells to near background levels.

FIG. 11 shows a similar inhibition of p24 antigen expression by TCS in HIV-infected monocyte/macrophages. Here the cells were exposed to 0.3 ug/ml TCS either at day zero in culture (closed circles) or after four days of culture (closed squares). As seen, the presence of TCS at time zero prevented expression of HIV antigen p24 in the cells, and TCS was also effective in reducing existing p24 levels to near zero four days after cell exposure to the inhibitor.

## B. Time Course of TCS and MMC Action

Cultured macrophages infected with HIV were treated with 5 ug/ml of TCS or MMC as in A above. At 1 and 4 days after addition of the drug, the cells were examined for expression of p24 antigen, by the above indirect immunofluorescence method. The data plotted in FIG. 12 show that about 2/3 of inhibition of p24 antigen expression occurs within 24 hours for both TCS (open triangles) and MMC (open circles). RT activity, measured as above, was also markedly decreased after 24 hours in cells treated with either drug. By day four, both p24 expression and RT activity were reduced to background levels, whereas untreated, HIV-infected cells showed no decrease in p24 expression (closed circles in FIG. 11) and no decrease in RT activity. Cell viability, as measured by the trypan blue exclusion test, was reduced to between about 60-70% during the four-day treatment with either drug.

## C. Dose Response to TCS and MMC

TCS or MMC was added to cultures of the infected cells at dose levels of either 0.5 or 5 ug/ml. The cells were examined for p24 expression four days after addition of the selected drug, as above. The results are plotted in FIG. 13. As seen, both drug concentrations gave nearly complete inhibition of p24 expression four days after addition of either TCS (triangles) or MMC (open circles).

To determine the response of the infected cells to lower concentrations of scRIP, the infected cells were exposed to MMC concentrations ranging from 0.005 to 5 ug/ml, for 3 hours. MMC was then washed out, and the cells were examined for inhibition of p24 expression 4 days after initial exposure to the scRIP. The results are shown in FIG. 14. Inhibition of HIV antigen expression was greater than 80% even at 0.005 ug/ml.

## D. Selective Inhibition of HIV Antigen Expression

To test whether inhibition of HIV p24 antigen expression mediated by TCS in vitro would be specific for HIV proteins, a standard HIV inhibition assay with

chronically infected macrophages was performed. In this experiment,  $1.5 \times 10^5$  macrophage per well were treated with various concentrations of TCS ranging from 5 nanograms per ml to 5 micrograms per ml for three hours, at which time free TCS was washed out of the culture. Treated and control cells were evaluated 4 days later. As shown in earlier experiments, pulse treatment of HIV infected macrophages with TCS substantially reduces the level of p24 protein expression even at a TCS concentration of 50 nanograms per ml. In the present study, the effect of TCS exposure on expression of cellular proteins and to a specific cellular antigen, HLA-DR, was also examined.

HLA-DR is normally detected on the surface of HIV infected macrophages at approximately 450 fluorescein units per cell (using directly fluoresceinated anti-DR monoclonal antibody as a staining reagent). No substantial decrease in the amount of DR was detected on the surface of HIV infected macrophages with TCS treatment. Even with TCS at a concentration of 5 ug/ml, there was no demonstrable decrease in the amount of cell surface DR antigens detected post-treatment, whereas HIV p24 decreased in a parallel assay by more than 70%, with measurable anti-HIV p24 effects of a 40% inhibition detected at 50 mg/ml. Similarly, parallel cultures treated with TCS were pulsed for 3 hrs with 3H-leucine (20 uCi/ml) 4 days after exposure to the TCS and radioactivity incorporated into protein was determined by TCA precipitation. No inhibition of 3H-leucine uptake was seen at any dose of TCS.

## EXAMPLE 8

Inhibition of Cellular Proliferation of HIV-Infected T Cells by TCS and MMC

## A. Cellular incorporation of 3H-thymidine

Noninfected H9 T cells or cells chronically infected with HIV, as above, were seeded in 96-well plates ( $2.5 \times 10^4$  cells per well in a total final volume of 200 ul of RPMI-1640 supplemented with 10% (v/v) heat inactivated fetal calf serum. TCS or MMC were added to yield final concentrations ranging from 0.01 to 1 ug/ml. After a three-day incubation at 37° C. in a humidified incubator supplied with 5-7% CO<sub>2</sub>, cultures were pulsed for 12 hours with 1 uCi/well of 3H-thymidine. After the 12 hour pulse period, cultures were harvested onto glass fiber filters by washing the plate wells with distilled water, and the filters were counted by liquid scintillation counting. Data are expressed as percent inhibition of radiolabel incorporation, relative to positive controls containing no test compound.

The data obtained for TCS exposure are plotted in FIG. 15 for noninfected (H9) and HIV-infected (H9.HIV) cells. The TCS concentration corresponding to the intersection between each curve and the 50% inhibition line indicates the TCS concentration effective to cause 50% inhibition of cellular proliferation, as evidenced by a 50% inhibition of thymidine uptake into DNA. The ratio of TCS concentrations needed to produce 50% inhibition in noninfected and HIV infected cells is defined as the selectivity index, and for TCS, is between about 50-60 in the continuous presence of the inhibitor. That is, approximately 50-60 times more TCS is required to produce a comparable level of inhibition in noninfected cells as in HIV-infected T cells. A similar selectivity index was observed for MMC. These selectivity indices were obtained in the continuous presence

of scRIP. Greater selectivity is observed with pulsed exposure, as described below. Comparable selectivity indices for  $^3\text{H}$ -leucine incorporation into noninfected and infected T cells, for both TCS and MMC treatment, were also observed.

#### B. Time of TCS and MMC Exposure

Noninfected H9 T cells or cells chronically infected with HIV were treated with TCS or MMC at 2  $\mu\text{g}/\text{ml}$ . At various time points (5, 15, 30, 60, 120 minutes) of exposure, aliquots of treated cells were washed to remove free TCS and MMC, and the cells were seeded in 96-well microtiter plates, as above, at  $2.5 \times 10^4$  cells/well. Cells were cultured for three days, then pulsed for 12 hours with 1  $\mu\text{Ci}/\text{well}$  of  $^3\text{H}$ -thymidine. After the 12 hour pulse period, cultures were harvested.  $^3\text{H}$ -thymidine incorporation into cellular DNA was measured by scintillation counting as above. Data are expressed as percent inhibition of radiolabel incorporation, relative to positive controls containing no test compound.

The data obtained for TCS and MMC exposure are plotted in FIGS. 16A and 16B, respectively. As seen, exposure to TCS or MMC for 1 hour or more produces greater than 50% inhibition of cellular proliferation in infected cells, whereas in noninfected cells, no significant inhibition was observed even after two hours of exposure to the added scRIP. Thus with limited (as opposed to continuous) exposure to the inhibitor, the selective inhibition of cellular proliferation of HIV-infected cells (Example 8A) is further enhanced.

#### C. Effects on cell growth and viability

Cell growth and viability were assessed 2 and 5 days after addition of anti-HIV agents. Cell counts were performed using a hemocytometer and viability was determined by trypan blue exclusion. As shown in FIGS. 17A and 17B, TCS and MMC treatment resulted in a concentration dependent inhibition of cellular proliferation (absolute count of viable cells/ml) at both days 2 and 5, with a preferential inhibition of the proliferation of HIV infected cells. FIG. 17C demonstrates that the percentage of viable cells in the treated cultures was decreased in a concentration dependent manner, with preferential effects on the HIV infected cells. Thus, the growth and viability of the cells appear to be impaired by both cytotoxic (decreased percentage of viable cells) and cytostatic (decreased absolute cell counts and decreased percentage of viable cells) mechanisms.

#### EXAMPLE 9

##### Effect of scRIP on Cellular Proliferation in HIV-Infected and noninfected T cells

The effect of several selected scRIP compounds on cellular proliferation in noninfected and HIV-infected T cells was examined, substantially according to the radiolabel uptake methods described in Example 8A. Specifically, cells were seeded in 96-well plates, then exposed to a selected concentration of the scRIP being tested for a three-day incubation period. The cells were then exposed to  $^3\text{H}$ -thymidine or  $^3\text{H}$ -leucine, at radiolabel concentrations of 1  $\mu\text{Ci}/\text{well}$ , for 12 hours, before harvesting the cells and counting incorporated radiolabel. As above, data are expressed as percent inhibition of radiolabel incorporation, relative to positive controls containing no test compound.

FIG. 18A shows the selective inhibition of cellular proliferation, effected by PAP-I treatment as evidenced

by thymidine incorporation in noninfected and HIV-infected T cells. The selectivity index, as defined above, is between 50-60. FIG. 18B shows a similar selective inhibition by PAP-I of leucine incorporation into infected and noninfected T cells. FIG. 19 shows a similar result for thymidine incorporation in infected and noninfected T cells exposed to PAP-II.

FIGS. 20-22 are similar plots of inhibition of thymidine incorporation into infected and non-infected cells exposed to mitogillin (FIG. 20), restrictocin (FIG. 21) and alpha-sarcin (FIG. 22). In each case, 50% percent inhibition of thymidine uptake into HIV-infected cells occurred at scRIP concentrations of about 0.05  $\mu\text{g}/\text{ml}$ , and the selectivity index was about 100.

#### EXAMPLE 10

##### Inhibition of Cellular Proliferation of HTLV-I-Infected T Cells by TCS and MMC

##### A. Cellular incorporation of $^3\text{H}$ -thymidine

HTLV-I infected producer cell line C91/PC was obtained from Dr. Gregory Reyes, Genelabs Incorporated (Redwood City, Calif.). H9 cells, a continuous cell line not infected with any known retrovirus served as a negative control cell line.

Noninfected H9 T cells or C91/PC cells chronically infected with HTLV-I were seeded in 96-well plates as in Example 7A. TCS, MMC, or mitogillin were added to yield final concentrations ranging from 0.05 to 5  $\mu\text{g}/\text{ml}$ . After a three-day incubation at  $37^\circ\text{C}$ . in a humidifier incubator supplied with 5-7%  $\text{CO}_2$ , cultures were pulsed for 12 hours with 1  $\mu\text{Ci}/\text{well}$  of  $^3\text{H}$ -thymidine. After the 12 hour pulse period, cultures were harvested onto glass fiber filters, and thymidine incorporated into cellular DNA was measured as above. Data are expressed as percent inhibition of radiolabel incorporation, relative to positive controls containing no test compound.

The data obtained for TCS exposure are plotted in FIG. 23A for noninfected (H9) and HTLV-I-infected (C91/PL) cells. As seen, there is no significant difference in cellular proliferation, as measured by thymidine incorporation, between infected and non-infected cells, at any concentration of TCS. Non-selective inhibition of cellular proliferation at increasing concentrations of TCS were observed.

Similar results were obtained for MMC (FIG. 23B) and mitogillin (FIG. 23C), showing no selective inhibitory effect on HTLV-I-infected cells, and a nonselective increase on cellular proliferation with increasing concentrations of either scRIP.

Although the invention has been described with respect to specific embodiments, uses and methods, it will be recognized that various changes, and modifications may be made without departing from the invention.

It is claimed:

1. A method of inhibiting HIV infection in human T lymphocyte cells and mononuclear phagocytic lineage cells infected with HIV, comprising

exposing the infected cells to a single-chain ribosome inactivating protein, at a concentration of the inactivating protein which is effective to inhibit viral antigen expression in the HIV-infected cells.

2. The method of claim 1, wherein said exposing is carried out at a concentration of the inactivating protein and for a duration which is effective to inhibit expression of HIV p24 antigen.

3. The method of claim 1, wherein said exposing is carried out at a concentration of the inactivating protein and for a duration which is effective to selectively inhibit expression of HIV antigens in HIV-infected mononuclear phagocytic lineage cells, as evidenced by a decrease in the ratio of a selected HIV antigen to a selected cellular antigen.

4. The method of claim 1, wherein said inactivating protein is selected from the group consisting of plant-derived single-chain ribosome inactivating proteins, and fungal-derived single-chain ribosome inactivating proteins.

5. The method of claim 4, wherein said inactivating protein is selected from the group consisting of pokeweed antiviral protein, alpha-sarcin, mitogillin, and restrictocin.

6. The method of claim 1, wherein the concentration of ribosome inactivating protein to which the infected cells are exposed is between about 0.01 and 5 ug/ml.

7. A method of selectively inhibiting cellular proliferation of HIV-infected T cells, comprising exposing the infected cells to a single-chain ribosome inactivating protein, at a concentration of the inactivating protein which is effective to selectively reduce the viability of HIV-infected T cells with respect to noninfected T cells.

8. The method of claim 7, wherein said inactivating protein is selected from the group consisting of plant-derived single-chain ribosome inactivating proteins, and fungal-derived single-chain ribosome inactivating proteins.

9. The method of claim 8, wherein said inactivating protein is selected from the group consisting of pokeweed antiviral protein, alpha-sarcin, mitogillin, and restrictocin.

10. The method of claim 7, wherein the concentration of ribosome inactivating protein to which the infected cells are exposed is between about 0.01 and 5 ug/ml.

11. A method of selectively inhibiting HIV antigen expression in HIV-infected mononuclear phagocytic lineage cells comprising

exposing the infected cells to a single-chain ribosome inactivating protein, at a concentration of the inactivating protein and for a duration which is effective to decrease in the ratio of a selected HIV antigen to a selected cellular antigen in the infected cells.

12. The method of claim 11, wherein the concentration of ribosome inactivating protein to which the in-

fect cells are exposed is between about 0.005 and 1 ug/ml.

13. The method of claim 12, wherein said inactivating protein is selected from the group consisting of plant-derived single-chain ribosome inactivating proteins, and fungal-derived single-chain ribosome inactivating proteins.

14. The method of claim 13, wherein said inactivating protein is selected from the group consisting of pokeweed antiviral protein, alpha-sarcin, mitogillin, and restrictocin.

15. A method of treating a human subject infected with HIV, comprising

administering a single-chain ribosome inactivating protein to the subject, at a dose which is effective to produce a measurable decrease in at least one of the following indications of HIV infection:

- (a) HIV antigen levels associated with HIV-infected T cells or mononuclear phagocytic lineage cells;
- (b) HIV antigen levels in the bloodstream;
- (c) the reverse transcriptase activity associated with HIV-infected T cells or mononuclear phagocytic lineage cells;
- (d) the ratio of viability of HIV-infected to uninfected T cells; and
- (e) the ratio of a selected HIV antigen to a selected cellular antigen in HIV-infected mononuclear phagocytic lineage cells.

16. The method of claim 15, wherein said decrease in indication(s) is measurable within 1-5 days after said administration.

17. The method of claim 15, which further includes alternatively measuring the decrease in at least one of said indications, and repeating said administering, until the measured indication of HIV infection shows no further decrease.

18. The method of claim 15, wherein the ribosome inactivating protein is administered parenterally, at a dose which is effective to inhibit HIV antigen expression associated with HIV-infected T cells or mononuclear phagocytic lineage cells.

19. The method of claim 15, wherein said inactivating protein is selected from the group consisting of plant-derived single-chain ribosome inactivating proteins, and fungal-derived single-chain ribosome inactivating proteins.

20. The method of claim 19, wherein said inactivating protein is selected from the group consisting of pokeweed antiviral protein, alpha-sarcin, mitogillin, and restrictocin.

\* \* \* \* \*

**UNITED STATES PATENT AND TRADEMARK OFFICE**  
**CERTIFICATE OF CORRECTION**

**PATENT NO. : 4,869,903**

**DATED : September 26, 1989**

**INVENTOR(S) : Jeffrey D. Lifson et al**

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

On the title page,  
in the Assignee:

Change "Genelabs Incorporated, Redwood City, Calif."  
to (add to)---Genelabs Incorporated, Redwood City,  
Calif. and The Regents of the University of  
California, Berkeley, Calif.---

**Signed and Sealed this**  
**Fifth Day of March, 1991**

*Attest:*

**HARRY F. MANBECK, JR.**

*Attesting Officer*

*Commissioner of Patents and Trademarks*



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## United States Patent [19]

Eda et al.

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[45] Date of Patent: Jan. 27, 1998

[54] HIV MONOCLONAL ANTIBODY SPECIFIC FOR THE HTLV-III<sub>MN</sub> GP120 ENVELOPE GLYCOPROTEIN

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[21] Appl. No.: 253,030

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[63] Continuation-in-part of Ser. No. 723,916, Jul. 1, 1991, abandoned.

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Primary Examiner—Paula K. Hutzell

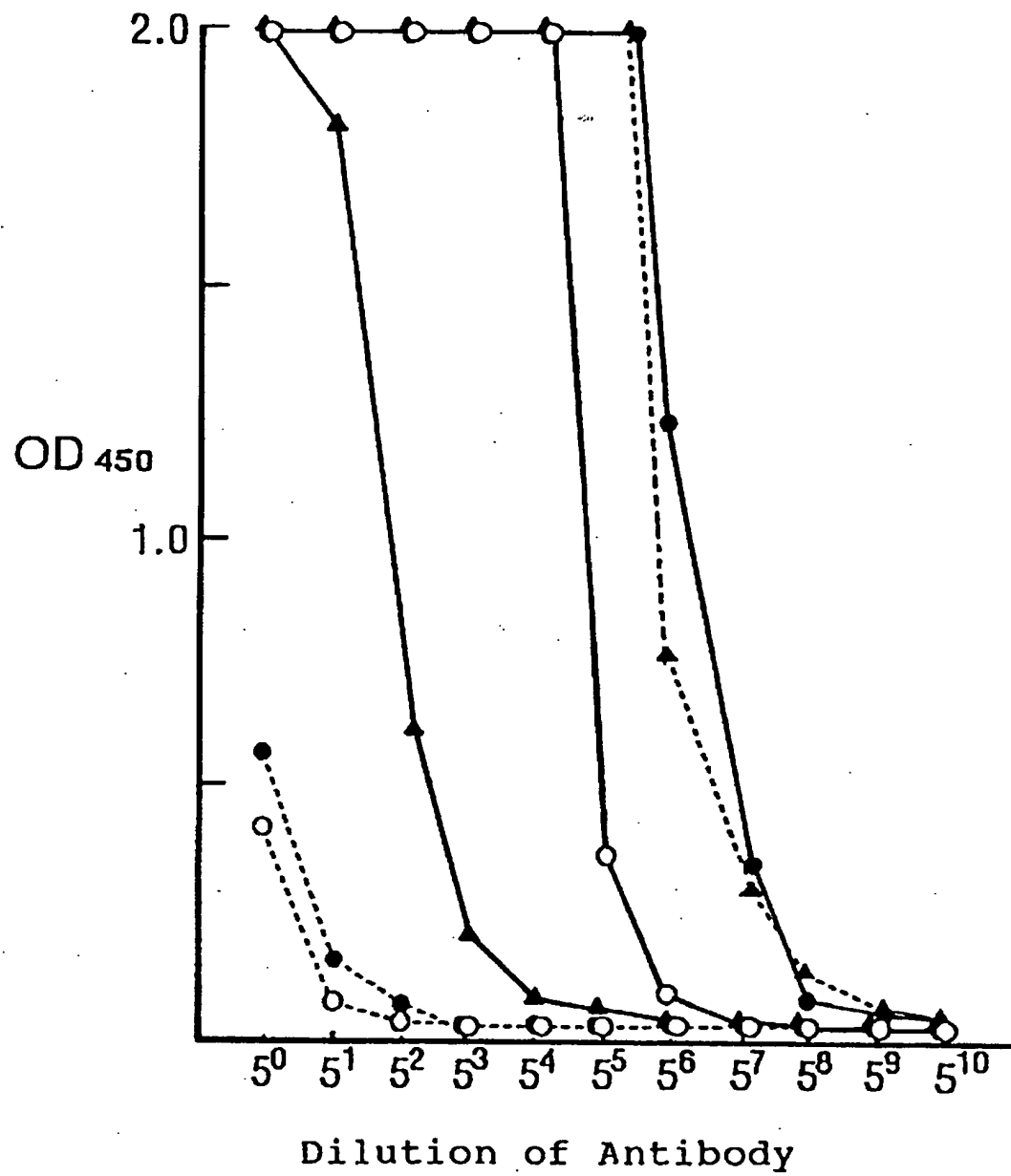
Attorney, Agent, or Firm—Foley &amp; Lardner

[57]

## ABSTRACT

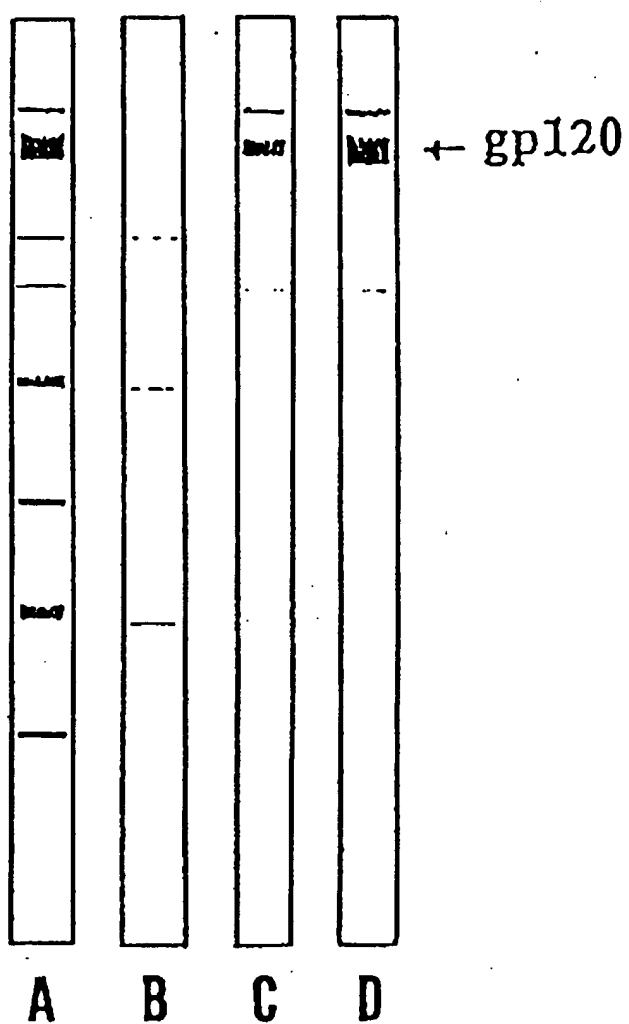
A murine monoclonal antibody which specifically binds to a glycoprotein antigen having a molecular weight of about  $12 \times 10^4$  daltons (gp120) present in the envelope of human T-lymphotropic virus III<sub>MN</sub> (HTLV-III<sub>MN</sub>) and capable of neutralizing the HTLV-III<sub>MN</sub> as determined by in vitro inhibition of syncytium formation but does not bind to other HTLV-III strains, or antigen-binding fragments, which is useful for prophylaxis, treatment and diagnosis of AIDS.

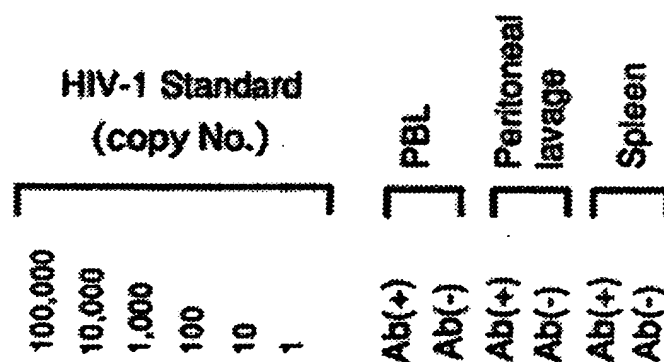
1 Claim, 5 Drawing Sheets

*FIG. 1*



**FIG. 2**



**FIG. 3**

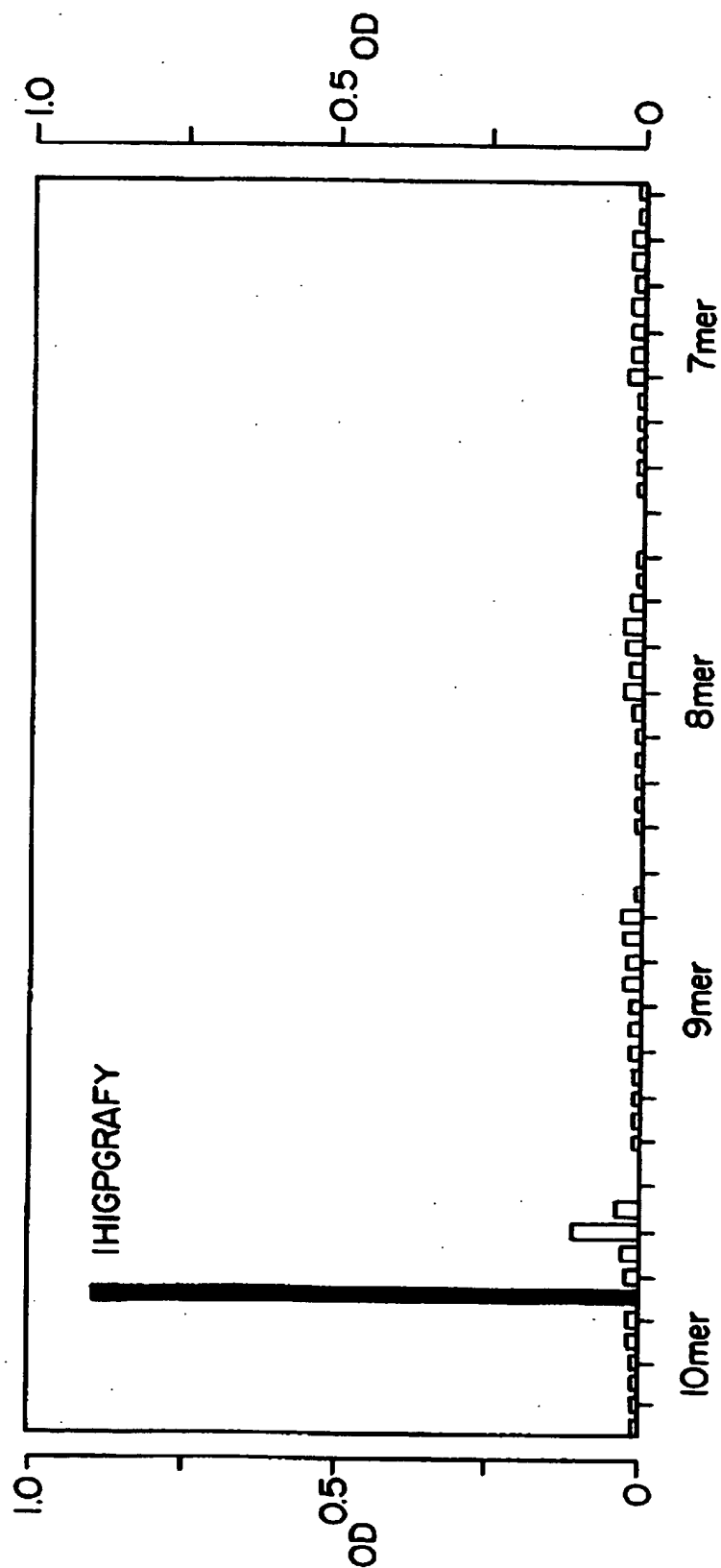
**FIG. 4A**

Figure 1 is a bar chart showing the optical density (OD) of various peptides. The y-axis is labeled 'OD' and ranges from 0 to 4. The x-axis is divided into five sections, each corresponding to a different peptide length: 15 mer, 14 mer, 13 mer, 12 mer, and 11 mer. Each section contains a list of peptides and their corresponding OD values.

Peptide Length	Peptide	OD (approx.)
15 mer	YNKRKRHIHIGPGRAF	2.2
	NKRKRHIHIGPGRAF	2.5
	KKRKRHIHIGPGRAF	2.8
	RKRKRHIHIGPGRAF	3.0
	KRIHIGPGRAF	3.2
	KRIHIGPGRAF	3.5
14 mer	KKRKRHIHIGPGRAF	2.5
	RKRKRHIHIGPGRAF	2.8
	KRIHIGPGRAF	3.0
	KRIHIGPGRAF	3.2
	KRIHIGPGRAF	3.5
13 mer	KKRKRHIHIGPGRAF	2.5
	RKRKRHIHIGPGRAF	2.8
	KRIHIGPGRAF	3.0
	KRIHIGPGRAF	3.2
	KRIHIGPGRAF	3.5
12 mer	KKRKRHIHIGPGRAF	2.5
	RKRKRHIHIGPGRAF	2.8
	KRIHIGPGRAF	3.0
	KRIHIGPGRAF	3.2
	KRIHIGPGRAF	3.5
11 mer	KKRKRHIHIGPGRAF	2.5
	RKRKRHIHIGPGRAF	2.8
	KRIHIGPGRAF	3.0
	KRIHIGPGRAF	3.2
	KRIHIGPGRAF	3.5

# HIV MONOCLONAL ANTIBODY SPECIFIC FOR THE HTLV-III<sub>MN</sub> GP120 ENVELOPE GLYCOPROTEIN

This application is a continuation-in-part application of U.S. Ser. No. 07/723,916 filed on Jul. 1 1991 (now abandoned).

This invention relates to an immunological technology providing a novel substance for the prophylaxis, treatment and diagnosis of virus infectious diseases. More particularly, it relates to a monoclonal antibody being capable of neutralizing human immunodeficiency virus (abbreviated as HIV) which is the etiologic agent of the acquired immunodeficiency syndrome (abbreviated as AIDS), and to a hybridoma being capable of secreting the monoclonal antibody.

## TECHNICAL BACKGROUND AND PRIOR ART

HIV is a retrovirus which is known to be cause of diseases such as AIDS and AIDS-related complex (abbreviated as ARC). It is well known that proto-type HIV includes human T-lymphotropic virus type III (abbreviated as HTLV-III) and lymphadenopathy associated virus (abbreviated as LAV). The above diseases are one of the recent most serious problems in the world, and it has been desired to develop a vaccine or a therapeutic method for the treatment thereof, but there has never been found any effective means. The most characteristic hematological anomaly in AIDS is functional and quantitative loss of helper/inducer T lymphocyte having CD4 antigen on the surface thereof. The immunodeficiency caused by HIV induces various disorders in the bio-phyllactic mechanism in the infected host (human), and then highly frequently induces opportunistic infections such as *Pneumocystis carinii* pneumonia and unusual malignant tumors such as Kaposi's sarcoma. The immunodeficiency caused by HIV is a progressive and irreversible disease with high death rate, and it is considered that the death rate of the disease will reach 100% within several years.

In case of infection of HIV to T cell with virus particles, the virus particles will first bind to the receptor CD4 antigen. The infection of HIV also spreads via cell-to-cell infection. That is, infected cells are cell-fused with non-infected cells, and particularly in organs such as brain, lymphonodus, etc., syncytium (macropolyocyte) is formed. The syncytium formation is also observed in experiment in vitro. It is usually considered that the T cells infected with HIV are easily suffered from cytopathic effect of HIV and this will cause the loss of CD4-positive cell.

It is also known that HIV infects not only the helper/inducer T lymphocytes but also monocyte/macrophages and further that most monocyte/macrophages and a part of the T lymphocytes have resistance to the cytopathic effect of HIV and hence these cells retain The virus for a long period of time and continuously produce the virus.

Moreover, it is known that human blood serum infected by HIV contains an antibody to HIV, but the antibody has merely low neutralizing activity (cf. Weiss et al., Nature, 316, p.69-72, 1985).

It is well known that a core antigen (gag) and an envelope antigen are present as the structural protein antigen of HIV. The HIV viral envelope protein is expressed as a precursor glycoprotein having a molecular weight of 160 kilodaltons (gp160) that is proteolytically cleaved to generate an external envelope glycoproteins having a molecular weight of 120 kilodaltons (gp120) and a trans-membrane envelope glycoprotein having a molecular weight of 41 kilodaltons (gp41). Among these, gp120 is the most important by the following reasons.

(1) When a test animal is infected with the gp120 or with a certain fragment derived from the gp120, a polyclonal neutralizing antibody is produced. This means that the gp120 is at least one of the target molecules of an antibody capable of neutralizing the virus (cf. Lasky et al., Science, 233, p.209-212, 1986).

(2) At the first step of infection of HIV, the gp120 binds to CD4 molecule of virus receptor. This means that the gp120 is the most important molecule as to the HIV infection (McDougal et al., Science 231, p.382-385, 1986).

(3) The syncytium formation by HIV, that is cell-to-cell infection of HIV, is induced by the direct interaction between the gp120 and the CD4 molecule of non-infected cells (cf. Lifson et al., Nature, 323, p.725-728, 1985).

Various monoclonal antibodies to constructive proteins of HTLV-III or LAV have been known, for example, antibody against p24 which is one of core antigens present within virus (Veronese, F. D., Proc. Natl. Acad. Sci., U.S.A., 82, p.5199-5202, 1985); antibody to pol gene product encoding a reverse transcriptase of virus (Veronese, F. D., Science, 231, p.1289-1291, 1986); and antibody to gp41 which is another constructive protein in the envelope (Veronese, F. D., Science, 229, p.1402-1405, 1985). However, none of these known monoclonal antibodies does react with the gp120 antigen which is an important factor for the prophylaxis and treatment of AIDS. It is rather reported that any monoclonal antibody being capable of effectively neutralizing the gp120 antigen could not be obtained even by immunizing animals with a purified LAV (Chassange, J. et al., J. Immunol., 136, p.1442-1445, 1985).

There have hitherto been studied various methods for obtaining monoclonal antibody being capable of effectively neutralizing AIDS virus and hence being useful for the prophylaxis, treatment and diagnosis of AIDS.

It is reported that a monoclonal antibody to the gp120 antigen has been obtained by using a synthetic peptide as an immunogen and that an epitope recognized by the antibody is within the region of the amino acid sequence 503-532 of the HIV envelope (Chanh, T. C. et al., Eur. J. Immunol., 16, p.1455-1468, 1986). However, the antibody had very weak binding activity as indicated both in Western blotting method and in immunofluorescent method. In this report, no evidence is shown of the presence of the neutralizing activity of said monoclonal antibody.

The present inventors have also obtained a monoclonal antibody (0.58) being capable of effectively neutralizing the virus by binding to the gp120 of HTLV-III<sub>B</sub> strain (Matsushita et al., J. Virology, 62, p.2107-2114, 1988). However, said 0.58 antibody can neutralize HTLV-III<sub>B</sub> strain, but not HTLV-III<sub>MN</sub> strain which is more popular in immunological field.

There has never been known any monoclonal antibody which can bind to the gp120 of the HTLV-III<sub>MN</sub> which is popular in immunological field and can substantially neutralize the virus.

## BRIEF DESCRIPTION OF THE INVENTION

The present inventors have found a monoclonal antibody which can bind to the envelope antigen of HTLV-III<sub>MN</sub>:gp120 and can substantially neutralize the virus.

An object of the invention is to provide a monoclonal antibody being capable of neutralizing HIV. Another object of the invention is to provide a hybridoma being capable of producing said monoclonal antibody. These and other objects and advantages of the invention will be apparent to those skilled in the art from the following description.

## BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows a reactivity of the monoclonal antibodies of the invention ( $\mu$ 39.1 and  $\mu$ 5.5) to the synthetic peptides of gp120 (amino acid sequence 303-325 or 308-329) derived from the various HIV mutants. An initial concentration of each antibody is 500  $\mu$ g/ml.

FIG. 2 shows a reactivity of the monoclonal antibodies of the invention ( $\mu$ 39.1 and  $\mu$ 5.5) to the external envelope glycoprotein gp120 derived from the HTLV-III<sub>MAN</sub>-infected cells.

FIG. 3 shows a protective effect of the monoclonal antibody  $\mu$ 5.5 on HTLV infection in mice with human peripheral blood lymphocyte (hu-PBL-SCID mice).

FIGS. 4A and 4B show the reactivity of overlapping peptides (SEQ ID NOS:1-23 and 25) of multiple lengths and the monoclonal antibody  $\mu$ 5.5 measured by Pepsacan (Peptide Scanning) method.

## DETAILED DESCRIPTION OF THE INVENTION

The term "neutralization" this disclosure means inhibition of cell free infection of HIV and also cell-to-cell infection such as syncytium formation which occurs between HIV-infected cells and non-infected cells by interaction between the gp120 and the CD4.

This invention provides a monoclonal antibody which can bind to a glycoprotein antigen having a molecular weight of about  $12 \times 10^4$  daltons present in the envelope of HIV and can neutralize said virus, and also fragments thereof.

The monoclonal antibody of this invention can recognize the envelope glycoprotein of HTLV-III<sub>MAN</sub> strain: gp120 and can neutralize said virus. The monoclonal antibody can be prepared by the following method.

A mammal (e.g. mouse, guinea pig, rabbit, etc.) is immunized with virus particles obtained from an appropriate HTLV-III<sub>MAN</sub>-producing cell or purified envelope glycoprotein gp120; a recombinant peptide prepared by a recombinant DNA technology, preferably a recombinant peptide corresponding to the amino acid sequence 247-370 of gp120; or a synthetic peptide prepared based on the amino acid sequence of the virus protein, preferably a synthetic peptide corresponding to the amino acid sequence 303-325 of gp120, more preferably corresponding to the amino acid sequence 309-318 of gp120. The spleen cells taken out from the thus immunized mammal is cell-fused with, for example, mouse myeloma cells to give a hybridoma, from which cells corresponding to a purified envelope glycoprotein gp120 or the above recombinant peptide or synthetic peptide are selected, and then the cells are cultivated to give the desired monoclonal antibody.

The above preparation of hybridoma can be carried out by a method of Kohler and Milstein (Nature, 256, p.495, 1975). The virus particles or envelope glycoprotein gp120 used as the antigen include HTLV-III<sub>MAN</sub>-producing cells prepared by sucrose density-gradient centrifugation method, e.g. derived from H9/HTLV-III<sub>MAN</sub>; a recombinant peptide prepared by a recombinant DNA technology; or a synthetic peptide prepared based on the amino acid sequence of said virus protein, and further any other immunogen prepared by a conventional method. The mouse to be immunized includes

BALB/c mouse, F1 mouse of BALB/c mouse and other mouse, and the like. Immunization is carried out by using an antigen of 20 to 200  $\mu$ g per one mouse (4 to 8 week age, weighing 20 to 30 g), wherein the antigen is administered 3 to 6 times for every 2 to 3 weeks. The feeding of mouse and the collection of spleen cell from the immunized mouse are carried out in a conventional manner.

Myeloma cells include MOPC-21NS/1 (Nature, 256, p.495, 1975), SP2/0-Ag14 (Nature, 276, p.269, 1979), p3X63Ag8-U1 (Eur. J. Immunol., 6, p.511, 1976), p3X63-Ag8 (Nature, 256, p.495, 1975), p3X63-Ag8.653 (J. Immunol., 123, p.1548, 1979), and the like.

The spleen cells and myeloma cells are mixed in a ratio of 1:1 to 10:1 by volume, and the cell-fusion is carried out in a phosphate buffer (pH 7.2-7.4) containing NaCl (about 0.85 wt. %), dimethylsulfoxide (10-20 v/v%) and polyethylene glycol having a molecular weight of 1,000 to 6,000, by incubating the mixture at 35° to 37° C. for 1 to 5 minutes. The fused cells (hybridoma) can be collected from the base medium containing hypoxanthine (1.3-1.4 mg/dl), aminopterin (18-20  $\mu$ g/dl), thymidine (375-4,000  $\mu$ l/dl), streptomycin (50-100  $\mu$ g/ml), penicillin (50-100 U/ml), glutamine (3.5-4.0 g/l) and fetal bovine serum (10-20 wt. %), wherein the fused cells grow. The base medium includes any medium which is usually used for cultivation of animal cells, such as RPMI1640 medium, Eagle's MEM medium, and the like. Cloning of the fused cells is repeated at least three times by limiting dilution method.

The hybridoma is cultivated in the same manner as usually used in cultivation of animal cells, whereby the desired monoclonal antibody of this invention is produced in the medium. For example, when the hybridoma ( $2 \times 10^6$ - $5 \times 10^6$  cells) is cultivated in RPMI1640 medium (10-20 ml) containing streptomycin (50-100  $\mu$ g/ml), penicillin (50-100 U/ml), glutamine (3.5-4.0 g/l) and fetal bovine serum (10-20 wt. %) in the presence of 5% CO<sub>2</sub> in a flask at 35°-37° C. for 3 to 7 days, whereby the antibody is secreted and accumulated in the medium. The hybridoma may also be grown by injecting intraperitoneally into a nude mouse or BALB/c mouse treated with pristane, whereby the antibody is accumulated within the ascites. That is, pristane (0.5-1 ml) is intraperitoneally inoculated into the mouse, and two to three weeks after the inoculation, the hybridoma ( $5 \times 10^6$ - $1 \times 10^7$  cells) is intraperitoneally transplanted thereto. After 7 to 10 days, accumulated ascites are collected. The monoclonal antibody contained in the culture medium or the ascites can be isolated by affinity chromatography with Affigel Protein A MAPS-II kit (BIO-RAD) or by any other conventional method.

The monoclonal antibody thus obtained can recognize an epitope on gp120 derived from HTLV-III<sub>MAN</sub> strain and can effectively neutralize the virus but does not bind to other HTLV-III strains. The monoclonal antibody has the following characteristics:

- (a) immunoglobulin class: IgG,  $\kappa$ ,
- (b) specifically binds to glycoprotein antigen having a molecular weight of  $12 \times 10^4$  daltons (gp120) of HTLV-III<sub>MAN</sub>,
- (c) specifically binds to an epitope which is present in the region represented by the amino acid sequence 303 to 325 (Tyr Asn Lys Arg Lys Arg Ile His Ile Gly Pro Gly

Arg Ala Phe Tyr Thr Lys Asn Ile Ile Gly) of gp120 of HTLV-III<sub>MN</sub> SEQ ID NO:24,

(d) specifically binds to the surface of HTLV-III<sub>MN</sub> viral particles and thereby inhibits the infection to CD4-positive cells by HTLV-III<sub>MN</sub>, and

(e) specifically binds to the surface of cells infected with HTLV-III<sub>MN</sub> and thereby inhibits the syncytium formation induced by interaction between the infected cells and uninfected cells as determined by in vitro inhibition of syncytium formation.

The monoclonal antibody of the present invention, more particularly, specifically binds to an epitope which is present in the region represented by the amino acid sequence 309 to 318 (Ile His Ile Gly Pro Gly Arg Ala Phe Tyr SEQ ID NO:25) of gp120 of HTLV-III<sub>MN</sub>.

Thus, the monoclonal antibody of this invention can clearly inhibit the cell-to-cell infection such as syncytium formation and/or cell-free virus infection such as infection with HTLV-III<sub>MN</sub>. Accordingly, the monoclonal antibody can be used for the prophylaxis and treatment of AIDS. Moreover, the monoclonal antibody of this invention is also useful for the inhibition of growth of AIDS virus in human host. Besides, since the monoclonal antibody of this invention has a strong neutralizing activity against HTLV-III<sub>MN</sub>, it is also effective for the prevention of infection of the virus to uninfected T cells.

A representative example of the hybridoma being capable of producing the monoclonal antibody of this invention has been deposited to Fermentation Research Institute, Agency of Industrial Science and Technology, Tsukuba, Japan under Budapest Treaty in accession No. FERM BP-3402 on Feb. 10, 1990.

This invention is illustrated by the following Examples but should not be construed to be limited thereto.

#### EXAMPLE 1

##### Preparation of monoclonal antibody:

##### Preparation of antigen

##### (1) A synthetic peptide:

A synthetic peptide corresponding to the amino acid sequence 303 to 325 of the envelope glycoprotein gp120 of HTLV-III<sub>MN</sub> (Tyr Asn Lys Arg Lys Arg Ile His Ile Gly Pro Gly Arg Ala Phe Tyr Thr Thr Lys Asn Ile Ile Gly SEQ ID NO:24) is used as an immunogen and an antigen for assay.

The above peptide is prepared with ABI430A Peptide Synthesizer (Applied Biosystem). The crude peptide thus prepared is removed from the substrate resin by TFMSA method (Yanaiharu, C., Experimental Medicine, 6, No. 10, p.141-148, 1988) and purified by reverse phase high performance liquid chromatography (HPLC). The purification by reverse phase HPLC is repeated three times and the fractions containing the product are collected, and the product is subjected to amino acid analysis, by which it is confirmed that the amino acid sequence of the product corresponds well to that of HTLV-III<sub>MN</sub> strain, and thereby it is concluded that the product is a synthetic peptide of gp120 of HTLV-III<sub>MN</sub> strain.

The thus-obtained synthetic peptide (designated "SP-1") is lyophilized, and then is bound to an immunization carrier, KLH (Keyhole Limpet Hemocyanin) to give a peptide-KLH conjugate in the following manner.

That is, the above peptide SP-1 (10 mg) is dissolved in 10 mM phosphate buffered saline (PBS, pH 7.0, 2 ml), and thereto is added a solution of MBS crosslinking agent in dimethylformamide (40 mg/100  $\mu$ l), and the mixture is stirred at room temperature for 30 minutes. The reaction mixture is washed with dichloromethane (2 ml) three times, and the aqueous layer (designated "Solution A") is separated.

Separately, KLH (20 mg) is dissolved in 0.2M Tris-HCl buffer (pH 8.6, 8M urea, 5 ml) and thereto is added dithiothreitol (DTT), and the mixture is stirred at room temperature for one hour. To the reaction mixture is added 10% trichloroacetic acid (3 ml), and the resulting precipitate is separated by filtration with suction, washed with distilled water (2 ml) and then dissolved in 20 mM sodium phosphate buffer (NaPB, pH 7.0, 0.6M urea, 5 ml) to give a solution (Solution B).

The above Solution A and Solution B are mixed and stirred at room temperature for 3 hours, and the reaction product is dialyzed and lyophilized.

The synthetic peptide of gp120 of HTLV-III<sub>MN</sub> strain and peptide-KLH conjugate prepared above are used as an immunogen and antigen for assay.

##### (2) Cultivation of HTLV-III<sub>MN</sub>-producing cells and preparation of HTLV-III<sub>MN</sub> particles:

H9/HTLV-III<sub>MN</sub> strain is used as HTLV-III<sub>MN</sub>-producing cells. A culture medium is RPMI 1640 supplemented with 20% FCS and 2 mM L-glutamine to be used in a 50 L scale. The H9/HTLV-III<sub>MN</sub> strain is cultivated in said culture medium in a 36 liter Spinner flask with a cultivation controller (manufactured by Wakenyaku Kogyo K.K.) and the resulting cells-floating mixture is centrifuged at 3,000 r.p.m. for 5 minutes to separate the culture supernatant. The culture supernatant is subjected to sucrose density-gradient centrifugation (25%, 50%, discontinuing, 89,000 $\times$ g, 20 hrs.) with a continuous rotator (RPC35T, manufactured by Hitachi Ltd.) at a rate of 2 liter/hr. to separate viral particles, wherein the viral particles are collected in 30-45% sucrose layer. The viral particles thus obtained are used as an immunogen and an antigen for assay.

Purified gp120 is prepared by collecting the cells from the above H9/HTLV-III<sub>MN</sub> culture broth by centrifugation, lysing the cells with 1% Triton X-100, centrifuging the mixture and then purifying the supernatant by affinity chromatography with ConA—Sepharose 4B column. The eluted solution is further purified by affinity chromatography with HIV antibody (IgG)—Sepharose 4B column. The purified gp120 thus obtained is used as an immunogen and an antigen for assay.

##### (3) Preparation of recombinant expression peptide of HTLV-III<sub>MN</sub> gp120 V3 domain:

H9/HTLV-III<sub>MN</sub> cells ( $10^6$ - $10^7$  cells) are floated in 1 $\times$ RSB buffer and thereto are added sodium dodecylsulfate (SDS, at final concentration of 1%) and Proteinase K (at final concentration of 1 mg/ml), and the mixture is incubated at 37 $^\circ$  C. for 2 hours. The resulting mixture is repeatedly subjected to extraction with phenol and precipitation with ethanol to give a high molecular weight DNA (genomic DNA). HTLV-III<sub>MN</sub> gp120 V3 domain (amino acid 247-370) is amplified by conventional PCR method by using a template of the above high molecular weight DNA and the following A primer (SEQ ID NO:26) and C primer (SEQ ID NO:27):

A primer: (5')GTGACACATGGAATTAGGCCAG(3')  
 C primer: (3')GAAGTCTCCCTGGGTCTTTA(5')

The amplification is carried out with Taq polymerase for 30 to 35 cycles.

The amplified DNA fragment is cloned with pUC18 plasmid, and the cloned DNA fragment is inserted into pUEX2 expression vector (manufactured by Amersham, code No. RPN1515; Bressan, G. and Stanley, Y., Nucleic Acid Research, 15, p.10056, 1987). *Escherichia coli* is transfected with the expression vector and then subjected to heat induction at 42° C. to express the peptide. The expressed HTLV-III<sub>MN</sub> gp120 V3 domain (amino acid 247-370) is a fusion protein with  $\beta$ -galactosidase, which is then purified in the form of *E. coli*-inclusion body as follows.

After expression, *E. coli* is fractured with glass beads and treated with lysozyme (final concentration, 0.1 mg/ml) at 4° C. The resulting precipitate separated by centrifugation is treated with Triton X-100 (final concentration, 0.5%). The precipitate is solubilized with 8M urea and is used as an immunogen and an antigen for assay.

#### Immuno-sensitization of mouse

An example of immuno-sensitization of mouse with the synthetic peptides prepared hereinabove is illustrated below.

BALB/c mice (4-8 weeks age) are inoculated with the synthetic peptide and synthetic peptide-KLH conjugated antigen mixture (each 100  $\mu$ g) three times in intraperitoneal route and one time in intravenous route, on the first day i.p. in the presence of Freund's complete adjuvant, on 14th day i.p. in the presence of Freund's incomplete adjuvant, on 28th day i.p. in the presence of Freund's incomplete adjuvant, and on 42nd day i.v. in the absence of an adjuvant.

#### Cell fusion and cultivation of hybridoma

Three days after the immunization, the spleen cells are collected from the mice in a usual manner.

The spleen cells are mixed with myeloma cells p3X63Ag8-U1 in a ratio of cells of 1:5, and the mixture is centrifuged (1,200 r.p.m./5 minutes) to remove the supernatant. The precipitated mass of cells is well untangled and is added to a mixture (1 ml) of polyethylene glycol-4000 (2 g), minimum essential medium (MEM) (2 ml) and dimethylsulfoxide, and the mixture is incubated at 37° C. for 5 minutes, and thereto is slowly added MEM so as to be total volume 50 ml. The mixture is centrifuged (900 r.p.m./5 minutes) to remove the supernatant fluid and the cells are untangled mildly. To the cells is added a normal medium (RPMI1640 medium with 10% FCA) (100 ml), and the cells are gradually suspended therein with a measuring pipette.

The suspension is poured into each well of a 24-well culture plate (1 ml/well) and the plate is incubated in an incubator containing 5% CO<sub>2</sub> at 37° C. for 24 hours. Then, 1 ml/well of HAT medium [a normal medium supplemented with hypoxanthine ( $1 \times 10^{-4}$ M), thymidine ( $1.5 \times 10^{-3}$ M) and aminopterin ( $4 \times 10^{-7}$ M)] is added and the plate is incubated for additional 24 hours. The culture is continued for 10 to 14 days in the same manner while exchanging the culture supernatant (1 ml) with the same volume of a HT medium (HAT medium depleted with aminopterin) every 24 hours for 2 days.

Each well with the fused cells (about 300 cells) growing in a colonial shape is selected. The culture supernatant (1 ml)

of the selected well is exchanged with the same volume of the HT medium and then the exchange is repeated every 24 hours for 2 days.

After 3 to 4 day culture with the HT medium, a part of the culture supernatant is collected and used for selection of the desired hybridoma by screening method as described hereinbelow.

#### Screening of hybridoma

The desired hybridoma is selected by a combination of enzyme immunoassay (EIA), immunofluorescence and Western blotting methods. The thus selected clone is measured for its neutralizing activity.

#### (1) EIA:

To each well of a 96-well microtest plate is added 100  $\mu$ l/well of the synthetic peptide antigen, purified gp120 antigen, or recombinant peptide (protein concentration: 2  $\mu$ g/ml), prepared as mentioned above, and the plate is incubated at 4° C. overnight for immobilization. Then, 2% bovine serum albumin (BSA) solution (100  $\mu$ l) is added to each well and the plate is incubated in the same manner for masking. To each well of the thus prepared antigen-immobilized plate are added the hybridomas obtained by the cell fusion and the culture supernatant of hybridomas after cloning and the plate is incubated at 37° C. for 2 hours. The plate is washed with 0.1% Tween 20/PBS three times and 100  $\mu$ l/well of a solution of peroxidase-labelled anti-mouse immunoglobulin (manufactured by Cappel,  $\times 5,000$  dilution). After incubation at 37° C. for 1 hour, the plate is washed with 0.1% Tween 20/PBS five times. Then, a substrate solution of 3,3',5,5'-tetramethylbenzidine (TMBZ) is added to each well for color development and an optical density is measured at 450 nm. A hybridoma clone is thus selected which strongly reacts only with the synthetic peptide derived from HTLV-III<sub>MN</sub> but not with the synthetic peptide derived from HTLV-III<sub>B</sub>.

#### (2) Immunofluorescence:

H9/HTLV-III<sub>MN</sub> cells or uninfected H9 cells ( $5 \times 10^5$  cells) suspended in the culture supernatant to be tested (100  $\mu$ l) are cultured at 4° C. for 30 minutes. The cultured cells are washed twice with a PBS solution containing BSA (2% and azide (0.1%) (PBS-BSA-Az). After washing, 100  $\mu$ l of anti-mouse IgG labelled with fluorescein-isothiocyanate (FITC) (manufactured by Sigma, diluted to 1:40 with PBS-BSA-Az) and the mixture is reacted at 4° C. for 30 minutes. The reaction mixture is washed with PBS-BSA-Az three times and then fixed with PBS containing 0.1% paraformaldehyde.

Using a laser flow-cytometry (Spectrum III manufactured by Ortho Diagnostics), the reactivity of the antibody is measured based on the strength of fluorescence. A hybridoma showing a maximum binding ability to the surface of H9/HTLV-III<sub>MN</sub> cells is selected and cloned by limiting dilution method. The hybridoma clone after cloning is also selected in the same manner.

#### (3) Western blotting:

Western blotting is carried out in accordance with Towbin et al. [Proc. Natl. Acad. Sci. U.S.A., 76, p.4350 (1979)].

A purified HTLV-III<sub>MN</sub> virus is prepared by the method described in the literature [Science, 224, p.497 (1984)] and electrophoresed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gel is then transferred to nitrocellulose membrane to transfer the



virus to the membrane and the membrane is cut into strips with 0.4 to 0.5 cm width. Each strip is immersed in a hybridoma culture supernatant and incubated at room temperature overnight. After washing with PBS three times, each strip is warmed in a solution of biotin-labelled anti-mouse IgG (manufactured by TAGO) diluted to 1:750. After washing with PBS three times, each strip is immersed in a solution of horseradish peroxidase-conjugated avidin (manufactured by Sigma) diluted to 1:1000 and warmed for 1 hour. After washing with PBS three times, a coloring reagent containing 4-chloro-1-naphthol (manufactured by Bio-Rad) is used for color development. A hybridoma showing a colored band of HTLV-III<sub>MAN</sub> gp120 is selected and cloned. The hybridoma clone after cloning is also selected in the same manner.

#### (4) Measurement of neutralizing activity:

The culture supernatant of H9/HTLV-III<sub>MAN</sub> is used as an original viral solution ( $10^{4.5}$  to  $10^5$  TCID<sub>50</sub>).

The viral solution adjusted to 10 TCID<sub>50</sub>/50  $\mu$ l and 50  $\mu$ l of the hybridoma clone culture supernatant or purified ascites, which are diluted in series, are inoculated into each well of a 96-well flat-bottomed plate and the plate is incubated at 37° C. for 1 hour. Then, MT4 cells are added to each well at  $10^4$  cells/100  $\mu$ l/well, said cells being floated in RPMI 1640 medium supplemented with 10% FCS, L-glutamine (3.5 to 4.0 g/l), penicillin (50 U/ml) and streptomycin (50 g/ml), and cultured at 37° C. for 5 days.

The neutralizing activity is evaluated based on an ability of the antibody to inhibit the syncytium formation observed during infection. The neutralization titer is expressed as a minimum effective concentration of the antibody showing 100% inhibition of syncytium formation.

The above selection procedure provides hybridomas ( $\mu$ 39.1 and  $\mu$ 5.5) capable of producing the desired monoclonal antibody.

Preparation of monoclonal antibodies with hybridomas  $\mu$ 39.1 and  $\mu$ 5.5:

Each  $5 \times 10^6$  cells/animal of the hybridoma  $\mu$ 39.1 or  $\mu$ 5.5 obtained above is intraperitoneally administered to pristane-treated female BALB/c mice (8 weeks age). After 10 to 21 days, ascites cancer is induced. Ascites are taken out from the mice and centrifuged at 3,000 rpm for 5 minutes to remove solid components. Then, the antibody is purified by subjecting the supernatant to affinity chromatography using Affigel Protein A MAPS-II Kit (manufactured by Bio-Rad).

### EXAMPLE 2

Analysis of monoclonal antibodies  $\mu$ 39.1 and  $\mu$ 5.5

(1) Reactivity to gp120 synthetic peptide derived from various HIV mutants:

Synthetic peptides of gp120 (amino acid sequence 303-325 or 308-329) derived from HTLV-III<sub>MAN</sub>, HTLV-III<sub>B</sub>, HTLV-III<sub>RF</sub>, and HIV-2 are employed. The reactivity is tested in the same manner as described in the above Screening of hybridoma, (1) EIA.

As shown in FIG. 1, it is clear that the control 0.56 antibody strongly reacts with the peptide derived from HTLV-III<sub>B</sub> but not with the peptide derived from HTLV-III<sub>MAN</sub> at a lower concentration although it cross-reacts with the peptide derived from HTLV-III<sub>MAN</sub> at a higher concentration.

On the other hand, it is seen that the monoclonal antibody  $\mu$ 39.1 is a HTLV-III<sub>MAN</sub>-specific antibody which strongly reacts with the peptide derived from HTLV-III<sub>MAN</sub>. It is also seen that the  $\mu$ 39.1 monoclonal antibody reacts neither with the synthetic peptides derived from HTLV-III<sub>RF</sub> nor with those from HIV-2 (data is not shown in FIG. 1).

The reactivity of the monoclonal antibody  $\mu$ 5.5 is completely the same as that of  $\mu$ 39.1, i.e. this monoclonal antibody is a HTLV-III<sub>MAN</sub>-specific antibody which strongly reacts only with the peptide derived from HTLV-III<sub>MAN</sub>.

(2) Reactivity to gp120 derived from infected cells (Western blotting):

In order to determine the reactivity of the monoclonal antibodies  $\mu$ 39.1 and  $\mu$ 5.5 to the external envelope glycoprotein gp120 derived from infected cells, Western blotting technique is used. An antigen used is an H9/HTLV-III<sub>MAN</sub> cell lysate. The procedure described in the above Screening of hybridoma, (3) Western blotting is repeated.

As shown in FIG. 2, strip A is a positive control in which HIV antibody positive human serum is employed, wherein a gp120 band is observed. The monoclonal antibody 0.56 does not react with gp120 derived from HTLV-III<sub>MAN</sub> (strip B) while the monoclonal antibodies  $\mu$ 39.1 and  $\mu$ 5.5 recognize gp120 derived from HTLV-III<sub>MAN</sub> (strips C and D). It is also found that the reactivity of the monoclonal antibody  $\mu$ 5.5 is stronger than that of the monoclonal antibody  $\mu$ 39.1 as shown in FIG. 2.

(3) Neutralizing property of monoclonal antibodies  $\mu$ 39.1 and  $\mu$ 5.5:

The neutralizing property of the monoclonal antibodies  $\mu$ 39.1 and  $\mu$ 5.5 is examined according to the procedure described in the above Screening of hybridoma, (4) measurement of neutralizing activity. The results are shown in the following Table 1.

TABLE 1

		Inhibitory activity on cell to cell infect. by infected cells <sup>1</sup>			Virus-neutralizing activity <sup>2</sup>		
		MoAb					
Virus		$\mu$ 5.5	$\mu$ 39.1	0.56	$\mu$ 5.5	$\mu$ 39.1	0.56
45	III <sub>MAN</sub>	16	63	>500	1	63	>500
	III <sub>B</sub> /LAV	>500	>500	31	>500	>500	4
	III <sub>RF</sub>	>500	>500	>500	>500	>500	>500
50							

(Note):

<sup>1</sup>Minimum effective concentration ( $\mu$ g/ml) of the antibody showing 80% inhibition of cell to cell infection by infected cells

<sup>2</sup>Minimum effective concentration ( $\mu$ g/ml) of the antibody showing 100% inhibition of viral infection

The right column in Table 1 shows a minimum effective concentration of the antibody showing 100% inhibition of infection of each variant viral species. The control monoclonal antibody 0.56 shows a neutralizing activity specific to HTLV-III<sub>B</sub>/LAV. On the other hand, the monoclonal antibody  $\mu$ 39.1 is a monoclonal antibody capable of specifically neutralizing HTLV-III<sub>MAN</sub> which inhibits the infection of HTLV-III<sub>MAN</sub> totally (100%) at a concentration of 63  $\mu$ g/ml but not the infection of the other HTLV strains III<sub>B</sub> and III<sub>RF</sub>. The monoclonal antibody  $\mu$ 5.5, likewise  $\mu$ 39.1, shows a neutralizing activity specific to the strain III<sub>MAN</sub>. It is seen

that the neutralizing activity of the monoclonal antibody  $\mu 5.5$  is more than 50 times higher than that of  $\mu 39.1$  and is a strong neutralizing antibody which inhibit the infection of the strain III<sub>MN</sub> totally (100%) at a concentration of 1  $\mu$ g/ml.

The left column of Table 1 indicates a minimum effective concentration of the antibody showing 80% inhibition of cell to cell infection by infected cells. The control monoclonal antibody 0.5 $\beta$  shows a neutralizing activity specific to III<sub>B</sub>/LAV infected cells. On the other hand, the monoclonal antibody  $\mu 39.1$  inhibits the cell to cell infection by III<sub>MN</sub> infected cells at a concentration of 63  $\mu$ g/ml but not the infection by III<sub>B</sub> or III<sub>RP</sub> infected cells. That is, it is found that the monoclonal antibody  $\mu 39.1$  is a neutralizing antibody specific to the strain III<sub>MN</sub> in the cell to cell infection by the infected cells.

The monoclonal antibody  $\mu 5.5$ , likewise  $\mu 39.1$ , also shows a neutralizing activity specific to the strain III<sub>MN</sub>. It is seen that the neutralizing activity of the monoclonal antibody  $\mu 5.5$  is more than about 4 times higher than that of  $\mu 39.1$  and is a strong neutralizing antibody which inhibit the cell to cell infection by the infected cells at a concentration of 16  $\mu$ g/ml.

#### EXAMPLE 3

Preventive effect of the monoclonal antibody  $\mu 5.5$  on HTLV infection in mice with human peripheral blood lymphocyte (hu-PBL-SCID mice)

$2 \times 10^7$  cells of human peripheral blood lymphocyte (PBL) having no infection with EB virus were administered to the peritoneal of severe complex immunodeficiency mice (SCID mice) of 7 to 15 weeks old. After 2 to 3 weeks, HTLV-III<sub>MN</sub> strain ( $5 \times 10^2$  TCID<sub>50</sub>) and the neutralizing antibody of the present invention ( $\mu 5.5$ ; 360 mg/kg) were simultaneously administered into the peritoneal of the SCID mice with human PBL. Each three mice were employed for the group that received the antibody of the invention and for the group that received no antibody.

Four days after the final administration, peripheral blood lymphocytes (PBL), peritoneal lavage cells and spleen were taken out, and the HTLV-III<sub>MN</sub> gene was detected and quantified by Polymerase Chain Reaction (PCR) method.

First, each of cells were floated in RSB buffer, thereto added SDS (final concentration 1%) and Proteinase K (final concentration 1 mg/ml), and the mixture was incubated at 37° C. for 2 hours. Thereafter, the procedures of phenol extraction and ethanol precipitation were repeated to give a high molecular weight DNA (genomic DNA). Using this high molecular weight DNA as a template, the gag region of HTLV-III<sub>MN</sub> was amplified by the PCR method with the following SK38/39 gag primers, followed by acrylamide gel electrophoresis. The gel after the electrophoresis was transferred to a nylon membrane, to which <sup>32</sup>P-labelled SK19 gag probe was hybridized to conduct autoradiography.

The results are shown in FIG. 3. In each of mouse PBL, peritoneally exudated cells and spleen, the group that received the administration of  $\mu 5.5$  antibody of the present invention exhibited inhibition of HTLV-III<sub>MN</sub> viral infection (reduction in an amount of detected viral genes) as compared to the group that received no antibody, which proves the protective effect of the antibody of the present invention on viral infection.

Spleen-derived cells ( $1 \times 10^6$  cells) were also cultured under stimulation with phytohemagglutinin (PHA) for 3 days and an amount of HTLV core antigen (p24) in the supernatant was measured. As a result, said p24 antigen (110 pg/ml) was detected in the group that received no antibody whereas the group that received the antibody remained negative (the amount of the p24 antigen: 10 pg/ml), which also proves the protective effect of the neutralizing antibody of the present invention on viral infection.

#### EXAMPLE 4

Analysis of epitope that is recognized by the monoclonal antibody  $\mu 5.5$

The V3 synthetic peptide antigen derived from HTLV-III<sub>MN</sub> strain used as an immunogen in the present invention has an amino acid sequence of YNKRKRIHIGPGRAFYTKNIIG (23 amino acids SEQ ID NO:24). The  $\mu 5.5$  monoclonal antibody of the present invention has been confirmed to react with the above 23 amino acid sequence. In order to determine which portion of this sequence the monoclonal antibody of the present invention recognizes, Epitope Analysis Method (Epitope Scanning Kit; Chiron Mimotopes Pty Ltd.) was used.

Overlapping peptides comprising 7 to 15 sequential amino acids from the above 23 amino acid sequence (i.e. a series of peptides having 7 to 15 sequential amino acids wherein each one amino acid is shifted from the N-terminus towards the C-terminus in each set of peptides) were synthesized on a polystyrene rod. The reactivity of these peptides with the  $\mu 5.5$  monoclonal antibody of the present invention was investigated by EIA for determining which portion of the 23 amino acid sequence is an epitope. As a result, it was found that the  $\mu 5.5$  monoclonal antibody of the present invention well reacted with peptides having at least 10 amino acids and containing IHIGPGRAFY (SEQ ID NO:25) but did not reacted peptides having less than 10 amino acids at all (data are shown in FIG. 4 SEQ ID NOS:1-23 and 25). From this, the ten amino acid sequence IHIGPGRAFY (SEQ ID NO:25) was found to be an essential epitope of the synthetic peptide which is recognized by the monoclonal antibody of the present invention.

SK38 (SEQ ID NO: 28); (5')ATAATCCACCTATCCAGTAGGAGAAAT(3')

SK39 (SEQ ID NO: 29); (5')TTTGGTCTTGCTTATGTCCAGAAATGC(3')

SK19 (SEQ ID NO: 30); (5')ATCCTGGGATTAAATAAATAGTAAGAATGTATAGCCTAC(3')

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

(iii) NUMBER OF SEQUENCES: 30

## (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 15 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Tyr Asn Lys Arg Lys Arg Ile His Ile Gly Pro Gly Arg Ala Phe  
 1 5 10 15

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 15 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Asn Lys Arg Lys Arg Ile His Ile Gly Pro Gly Arg Ala Phe Tyr  
 1 5 10 15

## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 15 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Lys Arg Lys Arg Ile His Ile Gly Pro Gly Arg Ala Phe Tyr Thr  
 1 5 10 15

## (2) INFORMATION FOR SEQ ID NO:4:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 15 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Arg Lys Arg Ile His Ile Gly Pro Gly Arg Ala Phe Tyr Thr Thr  
 1 5 10 15

## (2) INFORMATION FOR SEQ ID NO:5:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 15 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Lys Arg Ile His Ile Gly Pro Gly Arg Ala Phe Tyr Thr Thr Lys  
 1 5 10 15

## (2) INFORMATION FOR SEQ ID NO:6:

-continued

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 15 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Arg Ile His Ile Gly Pro Gly Arg Ala Phe Tyr Thr Thr Lys Asn  
 1 5 10 15

## (2) INFORMATION FOR SEQ ID NO:7:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Lys Arg Lys Arg Ile His Ile Gly Pro Gly Arg Ala Phe Tyr  
 1 5 10

## (2) INFORMATION FOR SEQ ID NO:8:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Arg Lys Arg Ile His Ile Gly Pro Gly Arg Ala Phe Tyr Thr  
 1 5 10

## (2) INFORMATION FOR SEQ ID NO:9:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Lys Arg Ile His Ile Gly Pro Gly Arg Ala Phe Tyr Thr Thr  
 1 5 10

## (2) INFORMATION FOR SEQ ID NO:10:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Arg Ile His Ile Gly Pro Gly Arg Ala Phe Tyr Thr Thr Lys  
 1 5 10

## (2) INFORMATION FOR SEQ ID NO:11:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Ile His Ile Gly Pro Gly Arg Ala Phe Tyr Thr Thr Lys Asn  
 1 5 10

-continued

## ( 2 ) INFORMATION FOR SEQ ID NO:12:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 13 amino acids
- ( B ) TYPE: amino acid
- ( C ) STRANDEDNESS:
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Arg Lys Arg Ile His Ile Gly Pro Gly Arg Ala Phe Tyr  
 1 5 10

## ( 2 ) INFORMATION FOR SEQ ID NO:13:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 13 amino acids
- ( B ) TYPE: amino acid
- ( C ) STRANDEDNESS:
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Lys Arg Ile His Ile Gly Pro Gly Arg Ala Phe Tyr Thr  
 1 5 10

## ( 2 ) INFORMATION FOR SEQ ID NO:14:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 13 amino acids
- ( B ) TYPE: amino acid
- ( C ) STRANDEDNESS:
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Arg Ile His Ile Gly Pro Gly Arg Ala Phe Tyr Thr Thr  
 1 5 10

## ( 2 ) INFORMATION FOR SEQ ID NO:15:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 13 amino acids
- ( B ) TYPE: amino acid
- ( C ) STRANDEDNESS:
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:15:

His Ile Gly Pro His Thr Ala Phe Tyr Thr Thr Lys Asn  
 1 5 10

## ( 2 ) INFORMATION FOR SEQ ID NO:16:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 12 amino acids
- ( B ) TYPE: amino acid
- ( C ) STRANDEDNESS:
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Ile Gly Pro His Arg Ala Phe Tyr Thr Thr Lys Asn  
 1 5 10

## ( 2 ) INFORMATION FOR SEQ ID NO:17:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 12 amino acids
- ( B ) TYPE: amino acid
- ( C ) STRANDEDNESS:
- ( D ) TOPOLOGY: linear

-continued

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Lys Arg Ile His Ile Gly Pro Gly Arg Ala Phe Tyr  
 1 5 10

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Arg Ile His Ile Gly Pro Gly Arg Ala Phe Tyr Thr  
 1 5 10

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

His Ile Gly Pro Gly Arg Ala Phe Tyr Thr Thr Lys  
 1 5 10

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Ile Gly Pro Gly Arg Ala Phe Tyr Thr Thr Lys Asn  
 1 5 10

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 11 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Arg Ile His Ile Gly Pro Gly Arg Ala Phe Tyr  
 1 5 10

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 11 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

Ile His Ile Gly Pro Gly Arg Ala Phe Tyr Thr  
 1 5 10

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 amino acids

-continued

(B) TYPE: amino acid  
(C) STRANDEDNESS:  
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

His Ile Gly Pro Gly Arg Ala Phe Tyr Thr  
1 5 10

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS:  
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

Tyr Asn Lys Arg Lys Arg Ile His Ile Gly Pro Gly Arg Ala Phe Tyr  
1 5 10 15

Thr Thr Lys Asn Ile Ile Gly  
20

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS:  
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

Ile His Ile Gly Pro Gly Arg Ala Phe Tyr  
1 5 10

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

TGACACATG GAATTAGGCC AG

22

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

ATTTCCTGGGT CCCCTCCTGA AG

22

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 28 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

ATAATCCACC TATCCCACTA GGAGAAAT

28

-continued

## ( 2 ) INFORMATION FOR SEQ ID NO:29:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 28 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:29:

TTTGGTCCTT GTCTTATGTC CAGAAATGC

28

## ( 2 ) INFORMATION FOR SEQ ID NO:30:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 41 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:30:

ATCCTGGGAT TAAATAAAAT AGTAAGAATG TATAGCCCTA C

41

## What is claimed is:

1. A murine monoclonal antibody which is produced by the hybridoma PERM BP-3402 and which is capable of neutralizing human T-lymphotropic virus III<sub>MN</sub> (HTLV-III<sub>MN</sub>) and has the following characteristics:

(a) immunoglobulin class: IgG, K,

(b) specifically binds to an epitope which is present in the region represented by the amino acid sequence 303 to 325 (Tyr Ash Lys Arg Lys Arg Ile His Ile Gly Pro Gly Arg Ala Phe Tyr Thr Thr Lys Asn Ile Ile Gly) of a glycoprotein antigen having a molecular weight of about  $12 \times 10^4$  daltons of gp120 of HTLV-III<sub>MN</sub>, SEQ ID NO:1,

25

(c) specifically binds to the surface of HTLV-III<sub>MN</sub> viral particles and thereby inhibits the infection of CD4-positive cells by HTLV-III<sub>MN</sub>,

30

(d) specifically binds to the surface of cells infected with HTLV-III<sub>MN</sub> and thereby inhibits the syncytium formation induced by interaction between the infected cells and uninfected cells as determined by in vitro inhibition of syncytium formation, and

35

(e) does not bind to any HTLV-III strain other than HTLV-III<sub>MN</sub>, or an antigen-binding fragment thereof.

\* \* \* \* \*





US005827723A

**United States Patent** [19]  
**Matsushita**

[11] **Patent Number:** **5,827,723**  
[45] **Date of Patent:** **Oct. 27, 1998**

[54] **NEUTRALIZING MONOCLONAL ANTIBODY  
0.5 $\beta$  WHICH BINDS AN EPITOPE LOCATED  
WITHIN THE REGION OF AMINO ACIDS  
308-331 OF HTLVIIIB GP120**

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Japan

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[63] Continuation of Ser. No. 618,033, Nov. 27, 1990, abandoned, which is a continuation of Ser. No. 198,957, May 26, 1988, abandoned.

[30] **Foreign Application Priority Data**

Jul. 29, 1987 [JP] Japan ..... 62-133909

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G01N 33/53

[52] **U.S. Cl.** ..... 435/240.27; 530/388.35;  
435/78.21

[58] **Field of Search** ..... 530/388.35; 435/172.2,  
435/70.21, 240.27, 7.21; 424/85.8, 148.1

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[57] **ABSTRACT**

The present invention relates to monoclonal antibody 0.5 $\beta$  which binds to an epitope located within the region of amino acids 308-331 of HTLVIIIB gp120 and is capable of substantially neutralising the activity of human immunodeficiency viruses, to a hybridoma which produces the 0.5 $\beta$  antibody, to processes for preparing them and to compositions containing an effective amount of the antibody.

**3 Claims, 4 Drawing Sheets**

FIG. 1A

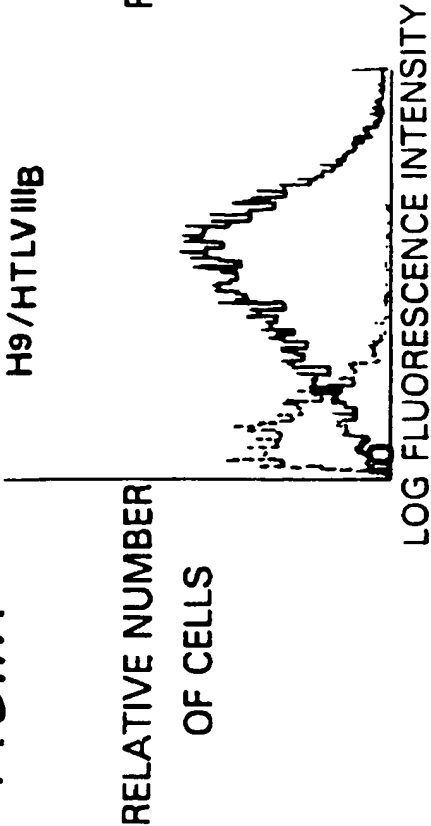


FIG. 1B

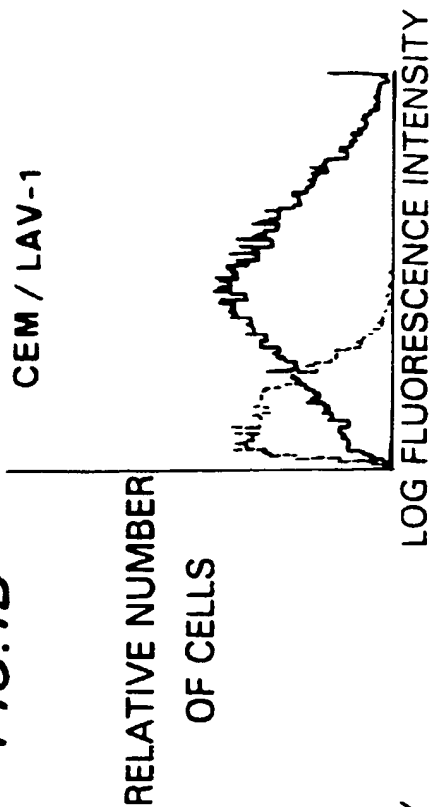


FIG. 1C

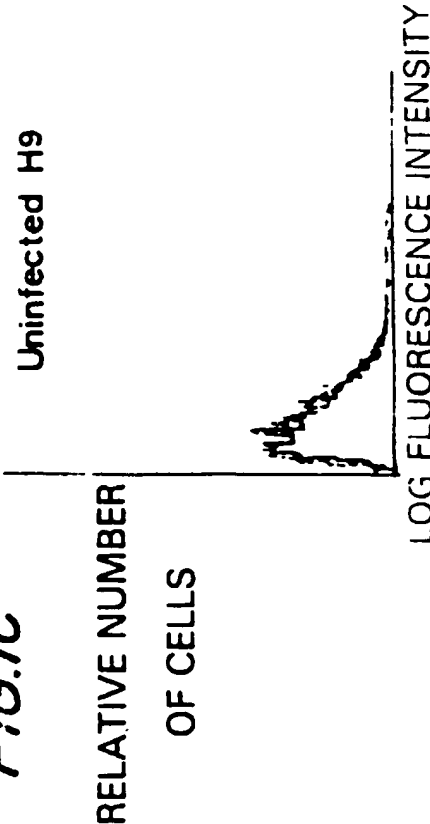
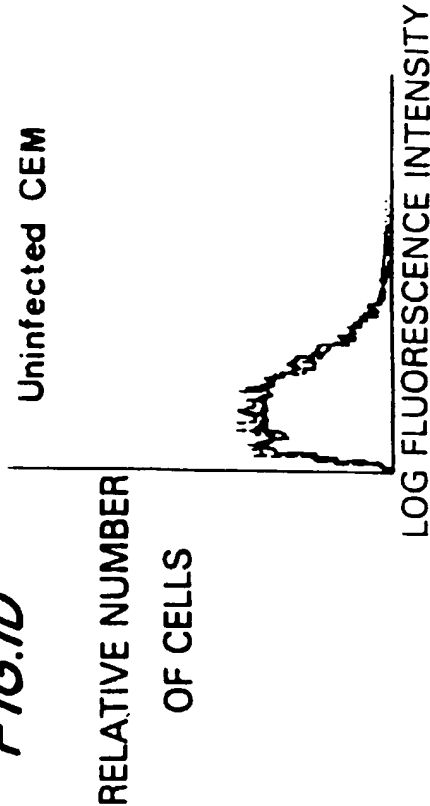
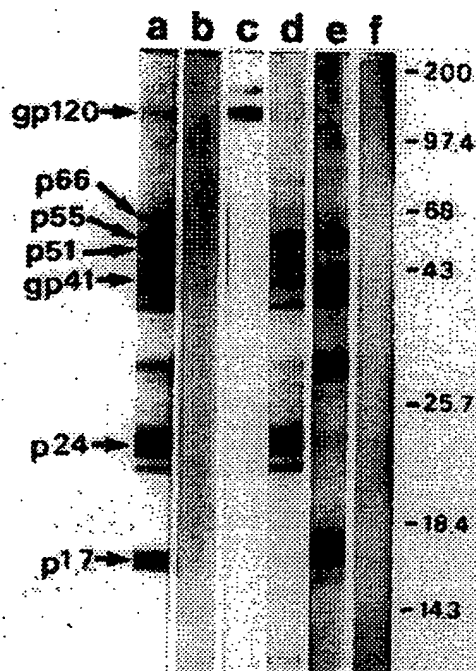
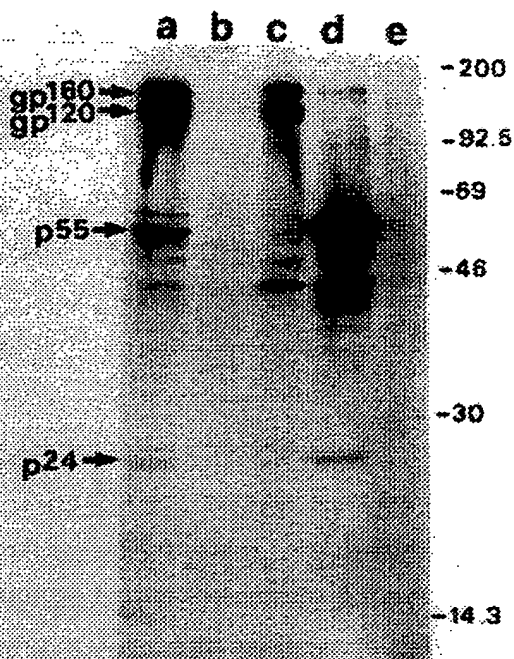
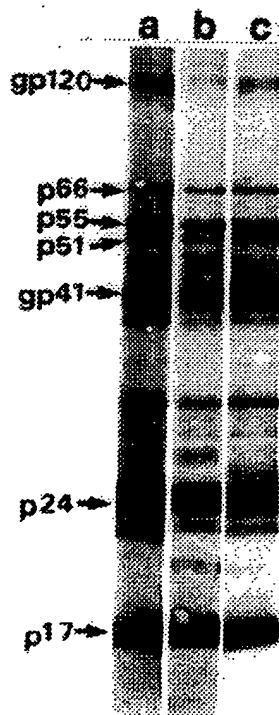
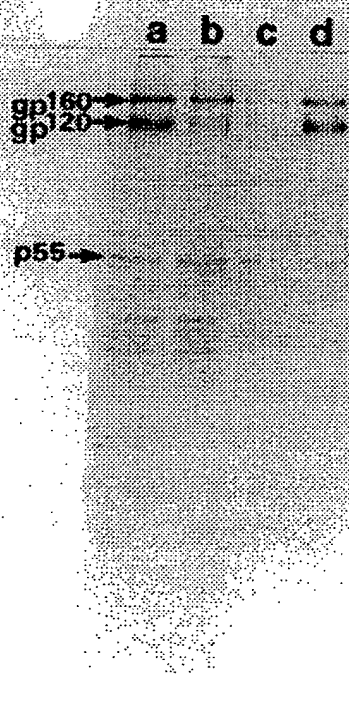
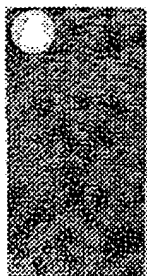
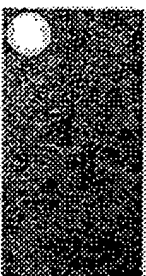
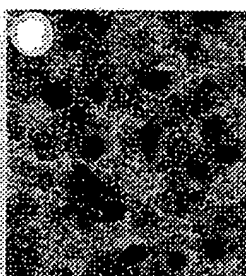
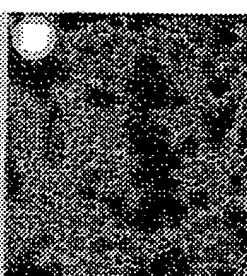
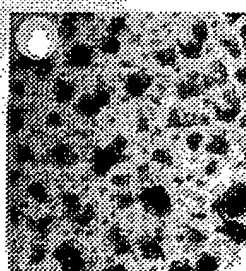
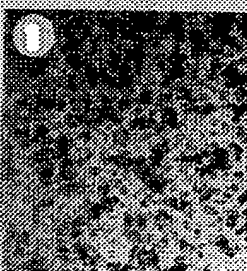
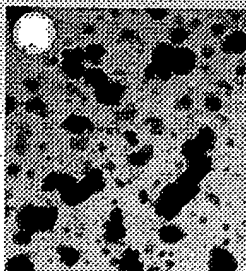
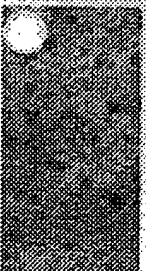
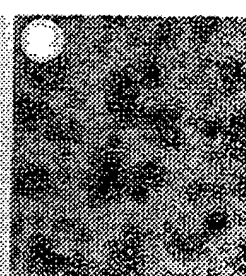
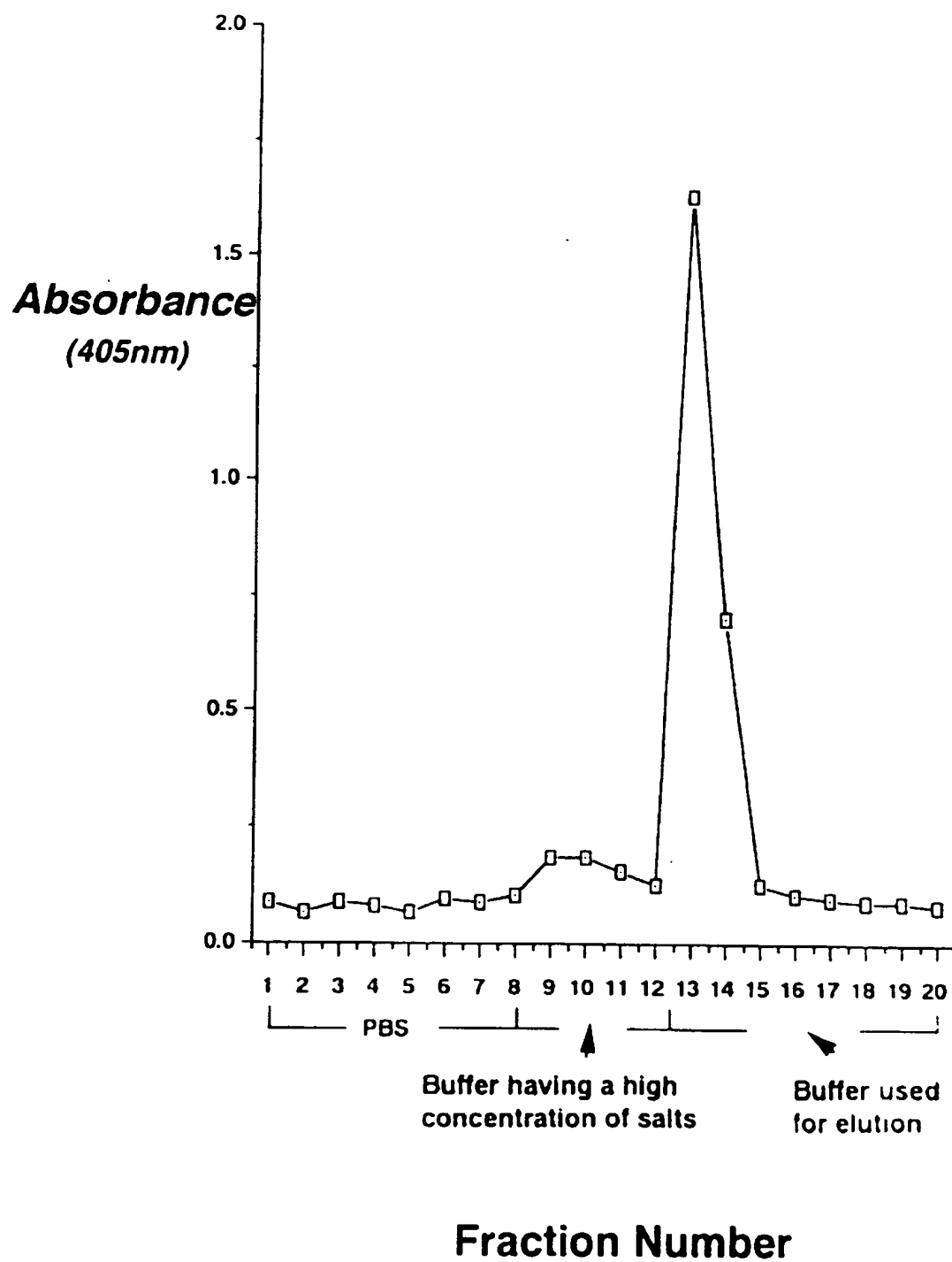


FIG. 1D



**FIG.2A****FIG.2B****FIG.2C****FIG.2D****FIG.2E**

*FIG. 3A**FIG. 3B**FIG. 3C**FIG. 3D**FIG. 3E**FIG. 3F**FIG. 3G**FIG. 3H**FIG. 3I**FIG. 3J*

**FIG. 4**

**NEUTRALIZING MONOCLONAL ANTIBODY  
0.58 WHICH BINDS AN EPITOPE LOCATED  
WITHIN THE REGION OF AMINO ACIDS  
308-331 OF HTLVIII GP120**

This is a continuing application of abandoned U.S. Ser. No. 618,033, filed on Nov. 27, 1990, now abandoned which is a continuation application of U.S. Ser. No. 198,957, filed on May 26, 1988, now abandoned.

**BACKGROUND OF THE INVENTION**

**1. Field of the Invention**

The present invention relates to monoclonal antibodies capable of neutralizing human immunodeficiency viruses.

**2. Description of the Prior Art**

Human immunodeficiency virus (HIV) is a human retrovirus which is an etiological factor for the Acquired Immunodeficiency Syndrome (AIDS) and related disorders such as Aids Related Complex (ARC). Nowadays, AIDS is well known as a world-wide epidemic, for which no effective vaccine or cure has yet been presented. The most profound hematologic feature associated with AIDS is the functional impairment and quantitative depletion of the helper/inducer subset of T-lymphocytes which express the CD4 surface antigen. HIV-induced immunosuppression results in a variety of deficiencies of the host defense system which render the body highly susceptible to opportunistic infections such as pneumocystic carinii pneumonia and unusual neoplasms such as Kaposi sarcoma. The immune defect appears to be progressive and irreversible and results in a very high mortality rate which is likely to approach 100% over a number of years.

In the first stage of infection of HIV to T cells, cell-free infection viz. attachment of cell-free virions to the target receptor CD4 antigen, occurs. However, HIV may also spread by cell-to-cell infection viz. by fusion of infected T cells with uninfected T cells so that the formation of syncytia (multi-nucleated giant cells) occurs in the organs such as the brain and the lymph nodes. Furthermore, the formation of syncytia may be observed in an in vitro assay. The depletion of CD4 positive cells may occur because the HIV-infected T cells are susceptible to the cytopathic effects of HIV.

It is known that HIV infects not only to the helper/inducer subsets of T cells but also to the cells of the monocyte/macrophage lineage. It was also known that most of monocytes/macrophages and certain T cells are resistant to the cytopathic effects of HIV and are thus considered to act as the reservoir cells of the viruses.

As is well known, prototype HIV are human T-lymphotropic virus type III (HTLV-III) and lymphadenopathy associated virus (LAV).

It is further known that polyclonal antibodies against HIV are present in sera obtained from HIV-infected humans, but the neutralizing activities of such antibodies are, in general, very weak. [reported, for example, by Weiss, R. A. et al, Nature, 316, 69-72 (1985)].

The existence of certain structural antigens of HIV including core (gag) antigens and envelope antigens are well known. The viral envelope comprises a 160 kilodalton (gp160) precursor glycoprotein which is subsequently cleaved into 120 kd (gp120) and 41 kd (gp41) glycoproteins present on the virion particle. The external envelope protein of HIV gp120 is the most important glycoprotein with respect to the following characteristics:

(1) Gp120 and/or certain fragments of gp120 are capable of inducing polyclonal neutralizing antibodies in experimental

animals. This means that gp120 is at least one of the target molecules of neutralizing antibodies [as disclosed, for example, in Lasky, L. A. et al. Science, 233, 209-212 (1986); Robey, W. G. et al. Proc. Natl. Acad. Sci. U.S.A., 83, 7023-7027 (1986) and Putney S. D. et al, Science, 234, 1392-1395 (1987)].

(2) The infection of HIV is initiated by binding of gp120 to the receptor CD4 molecule. This means that gp120 is a critical molecule for HIV with respect to the infection to target cells [as disclosed, for example, in McDougal J. S. et al, Science, 231, 382-385 (1986)].

(3) The formation of syncytia induced by HIV viz. the cell-to-cell infection of HIV depends on the direct interaction of gp120 with CD4 molecules of the uninfected cells [as disclosed, for example, in Lifson J. D. et al, Nature 323, 725-728 (1986)].

Various monoclonal antibodies against the protein components of HTLV-III or LAV have hitherto been proposed, as exemplified by those against p24, one of the core antigens present on the inside of the viruses [Veronese F. D., Proc. Natl. Acad. Sci. USA., 82, 5199-5202(1985); those against the product from the pol gene capable of coding the reverse transcriptase of the viruses [Veronese F. D. et al., Science 231, 1289-1291 (1986); and those against gp41, part of the envelope [Veronese F. D. et al. Science 229, 1402-1405 (1985)]. However, none of these antibodies are capable of reacting with gp120 antigen which is important for preventing and treating AIDS. It has been reported that no monoclonal antibody capable of effectively neutralizing gp120 was observed by immunization with purified LAV [for example, Chassigne J. et al, J. Immunol. vol. 136, 1442-1445 (1986)].

Various attempts have been made to provide monoclonal antibodies which are capable of effectively neutralizing AIDS viruses and which may be used for diagnosis and treatment of AIDS.

Recently, it has been reported that a monoclonal antibody capable of reacting with gp120 may be obtained by using a synthetic peptide as immunogen [Chanh T. C. et al, Eur. J. Immunol., 16: 1465-1468 (December 1986)]. The epitope recognized by this monoclonal antibody is located within amino acid sequence 503 to 532 of HIV gp160, the amino acid sequence of HIV being determined with reference to Ratner I. et al. Nature, 313: 277-284 (1985). However, the binding activity of this monoclonal antibody is weak when determined by the Western blot method and the surface immunostaining of HIV-infected cells. Moreover, no evidence of the neutralizing activity of this monoclonal antibody is shown in this report.

The present invention is based upon the discovery that the monoclonal antibodies which I have prepared are capable of significantly neutralizing (hereinafter defined) HIV by binding with an epitope of the HIV envelope antigens.

The present invention is directed to provide monoclonal antibodies capable of substantially neutralizing HTLV-III and LAV viruses and to hybridoma for the preparation of such monoclonal antibodies.

The term neutralizing used herein denotes the inhibition of HIV infection by cell-free virions and/or the inhibition of cell-to-cell infection such as the formation of syncytia by the fusion of HIV-infected cells with uninfected cells induced by the interaction of gp120 with CD4 molecules.

**SUMMARY OF THE INVENTION**

According to one feature of the present invention, there is provided a monoclonal antibody or fragment thereof capable of specifically binding to a glycoprotein antigen having a

molecular weight of 120,000 dalton and located at the envelope of human T-lymphotropic virus Type III (HTLV-III) and lymphadenopathy associated virus (LAV) and capable of substantially neutralizing said viruses.

Preferably the monoclonal antibody has the following characteristics:

- (a) classified into IgG<sub>1</sub> class;
- (b) capable of binding to the surfaces of a cell infected with HTLV-III virus thereby inhibiting the formation of syncytia induced from the infected cells and uninfected target T cells;
- (c) capable of binding to a precursor of a glycoprotein antigen having a molecular weight of 160,000 dalton;
- (d) capable of binding to an epitope located within amino acid sequence of 308 to 331 of HIV gp160, when determined with reference to Ratner, I., et al, *Nature*, 313, 277-284 (1985).

#### DESCRIPTION OF SPECIFIC EMBODIMENTS

The antibodies of the present invention may be prepared by immunizing a mammal, for example, a mouse, guinea pig or rabbit with a virus protein obtained from HTLV-III-producing cells, fusing the resultant spleen cells with myeloma cells of a mammal, for example, a mouse, and culturing the resultant hybridoma cells.

Although any and all cells which are capable of producing HIV when cultured may be used for the purpose of the present invention, it is preferred to use H9/HTLV-III<sub>B</sub> which is disclosed in JP-A-500767/86 (WO85/04897) and which was deposited with the American Type Culture Collection as ATCC CRL, No. 8543.

Culturing of HIV-producing cells and the recovery of HIV may be effected with reference, for example, to JP-A-285756/86 which disclose the characteristics and purification methods for the protein components of HIV, in particular, those of envelope protein.

For example, immunization of a mouse may be effected by administering hypodermically, intravenously or abdominally, purified HTLV-III viruses together with a suitable adjuvant, for example, Freund's complete adjuvant, aluminium hydroxide gel or pertussis vaccine.

HTLV-III may be administered 3-5 times at 0.05-0.1 mg per dose at intervals of 1-3 weeks. 3-7 days after the final immunization, the spleen is removed from the mouse in conventional manner.

Myeloma cell lines of mouse origin may be used for fusion. Examples of preferred cell lines include 8-azaguanine-resistant murine myeloma cell lines such as P3-X63-Ag8-U1 (P3-U1) from BALB/c strain [*European J. Immunology* 6, 511-519 (1976)]; SP2/0-Ag14 (SP-2) [*Nature* 276, 269-270 (1978)]; P3-X63-Ag8.653 (653) [*J. Immunology* 123, 1548-1550 (1979)]; P3-X63-Ag8 (X63) [*Nature* 256, 495-497 (1975)] and the like. The cell lines may be subcultured, for example using an 8-azaguanine medium viz. a medium prepared by adding 8-azaguanine (15 µg/ml) to a normal medium [prepared by adding to RPMI-1640 medium, glutamine (1.5 mM), 2-mercaptoethanol (5×10<sup>-5</sup>M), gentamycin (10 µg/ml) and 10% FCS (fetal calf serum: commercial product of CSL, Australia)].

The cells are further subcultured for 3-4 days before fusion using a normal medium so as to ensure that the number of cells is greater than 2×10<sup>7</sup> on the date of hybridization. Hybridization may be effected in conventional manner as follows:

The above-mentioned myeloma cells and the spleen cells are well washed with MEM medium or with PBS and mixed

together, the ratio of the number of spleen cells to the number of myeloma cells being preferably 5-10:1. The mixture is then centrifuged (1,200 r.p.m./5 min) to remove the supernatant. The pelleted cells are well loosened and then a mixed solution of polyethylene glycol 4000 (2 g), MEM medium (2 ml) and dimethyl sulfoxide (0.7 ml) is added in an amount of 0.2-1 ml per 10<sup>5</sup> antibody-producing cells at a temperature of 37° C. with stirring. MEM medium (1-2 ml at a time) is added several times to the cell suspension at intervals of 1-2 minutes. After this the cell suspension is made up to 50 ml in total by adding MEM medium. The supernatant is removed from the culture by centrifugation (900 r.p.m./5 min). The cells are loosened and normal medium (100 ml of RPMI-1640 containing 10% FCS) is added to the cells. The cells are resuspended by gentle pipetting.

The cell suspension is poured into each well of a 24-well culture Plate (1 ml/well) for incubation at a temperature of 37° C. for 24 hours in a 5% CO<sub>2</sub> incubator. To each well of the plate is then added HAT medium (1 ml) [prepared by adding hypoxanthine (10<sup>-4</sup>M), thymidine (1.5×10<sup>-5</sup>M) and aminopterin (4×10<sup>-7</sup> M) to the normal medium] to continue the culturing for 24 hours further. 1 ml of the supernatant is removed from each well and replaced by the same amount of fresh HAT medium at an interval of 24 hours for a period of 2 days. The culturing is continued further at a temperature of 37° C. for 10-14 hours in a CO<sub>2</sub> incubator. When the grown colonies of the fused cells are observed in certain wells, 1 ml of the supernatant is removed from each of these wells and replaced by the same amount of fresh HT medium [prepared by omitting aminopterin from HAT medium]. The replacement of the medium by HT medium is continued further for 2 days at an interval of 24 hours.

After further culturing for 3-4 days using HT medium, part of the supernatant is collected from the culture to determine the titre of antibody capable of binding to the surfaces of H9/HTLV-III<sub>B</sub> cells by the fluorescein antibody method as follows:

Fluorescein antibody method:

H9/HTLV-III<sub>B</sub> (H9/III<sub>B</sub>) cells or uninfected H9 cells (5×10<sup>5</sup>) are suspended in the supernatant of the test culture for incubating at a temperature of 4° C. for 30 minutes. The cells are washed twice with PBS containing BSA (2%) and azide (0.1%) (hereinafter referred to as PBS-BSA-Az). To the washed cells are then added 100 µl of anti-mouse IgG (Sigma), the IgG being labelled with FITC (fluorescein isothiocyanate) and diluted with PBS-BSA-Az at a ratio of 1:40. The mixture is incubated at a temperature of 4° C. for 30 minutes. After washing three times with PBS-BSA-Az, the reaction mixture is fixed using PBS containing paraformaldehyde (0.1%).

The antibody reactivity is determined by the use of laser flow cytometry (Spectrum III, commercial product of Ortho niagnostics Inc.) with reference to the fluorescence intensity of fluorescein.

A clone exhibiting the highest binding ability to the surfaces of H9/HTLV-III cells is selected and subcloned to obtain the desired hybridoma cells.

Hybridoma 54/CB1, a hybridoma cell exhibiting the highest productivity among the resultant hybridoma cells was deposited with the European Collection of Animal Cell Cultures located at Portondown, Salisbury, Wilts, England on 14th May 1987 and designated as 54/CB1 ECACC No. 87051401.

The preparation of monoclonal antibodies from the hybridoma may be effected, for example, as follows:

Pristane [2,6,10,14-tetramethylpentadecane: 0.5 ml] is abdominally given to a nude mouse (female; 8-10 weeks

old). Two weeks after the inoculation of the pristane-treated mouse, the hybridoma cells are abdominally administered in an amount of  $2-4 \times 10^6$  cells. 10–21 days after this, ascites tumour is induced by the hybridoma cells. The ascitic fluid is collected from the mouse and centrifuged (3000 r.p.m./5 min) to remove solids. After salting-out with ammonium sulfate (50%), the solution is subjected to dialysis against 0.04M phosphate-buffered solution containing 0.03M NaCl (pH 8.0). The resultant residue is passed through a column packed with DES2 (commercial product of Whatman, U.S.A.) to collect IgG fractions which may be used as purified monoclonal antibody. The isotype of the monoclonal antibody may be determined by Ouchterlony's method (double diffusion test) [see "Menekigaku Jikken Nyumon, Seibutu-kagaku Jikkenho 15, page 74 (1981)", published by Gakkai Shuppan Centre, Japan].

Quantitative determination of protein may be effected by the Folin's method and with reference to an optical density at 280 nm [ $1.4 (OD_{280}) \sim$ immunoglobulin 1 mg/ml].

The characteristics of the monoclonal antibody of the present invention may be determined by the Western blotting method, the radioimmunoprecipitation method, the cross-precipitation method and the detection of the digest pattern using endoglycosidase, as described in the example herein-after.

It has been confirmed that the monoclonal antibody of the present invention is capable of inhibiting both cell-to-cell infection, as identified by the formation of syncytia, and cell-free infection.

The monoclonal antibodies of the present invention may be used for the diagnosis, prevention and treatment of AIDS as well as for the purification of AIDS virus antigen.

Thus, it would be possible to inhibit growth of the viruses in human hosts or to inhibit further infection by the viruses by the use of the monoclonal antibody of the present invention, and also the monoclonal antibody of the present invention may be used for treating AIDS.

In this respect, the monoclonal antibodies of the present invention may be effective for preventing HIV infection of uninfected T4 cells due to their strong neutralizing ability.

Thus, the present invention further relates to a pharmaceutical composition comprising, in addition to one or more pharmaceutically inert excipients and/or carriers, an effective amount of at least one monoclonal antibody as hereinbefore described.

It is also possible to use the monoclonal antibodies of the present invention for purification of HIV envelope antigen, for example when bound to a suitable carrier material. The invention therefore includes within its scope an antibody-affinity carrier wherein a monoclonal antibody as hereinbefore described is bound to a suitable carrier material.

The following example illustrates the present invention in detail, wherein the treatments were effected at room temperature unless otherwise specified.

#### BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A shows the reactivity of the antibody of the present invention against H9 cells infected with HTLVIII<sub>B</sub>; FIG. 1B shows the reactivity of the antibody of the present invention against CEM cells infected with LAV-1; FIG. 1C shows the reactivity of the antibody of the present invention against uninfected H9 cells; FIG. 1D shows the reactivity of the antibody of the present invention against uninfected CEM cells;

FIG. 2 shows the Patterns of the antibodies of the present invention detected by electrophoresis. FIG. 2A shows the pattern detected by the Western blotting method, FIG. 2B

shows the pattern obtained by the radioimmunoprecipitation method, FIG. 2C and FIG. 2D show respectively the patterns detected by the cross-precipitation method, and FIG. 2E shows the pattern of digestion by endoglycosidase H;

FIG. 3 shows the inhibition of syncytia induced by H9/HTLVIII<sub>B</sub> cells by the 0.5 $\beta$  antibody. FIG. 3A and FIG. 3F are control panels of CEM cells alone; FIG. 3B and FIG. 3G are control panels of H9/HTLV-III<sub>B</sub> cells alone; FIG. 3C and FIG. 3H show syncytia resulting from preincubation with 50 ug of MOPC 21 per ml; FIG. 3D and FIG. 3I show syncytia resulting from preincubation with 50 ug of 0.5 $\beta$  antibody per ml; FIG. 3E and FIG. 3J show syncytia resulting from preincubation with 5 ug of 0.5 $\beta$  antibody per ml; and

FIG. 4 shows a pattern of elution obtained by the present antibody when used for purifying the envelope antigen of H9/HTLV-III<sub>B</sub> origin.

#### EXAMPLE

##### 1) Preparation of antigen:

H9 cells infected with HTLV-III<sub>B</sub> [Science 224, 497–500 (1984)] (hereinafter referred to as H9/HTLV<sub>B</sub>) were cultured using RPMI 1640 medium containing 10% FCS in an incubator containing 5% CO<sub>2</sub> at a temperature of 37° C. for 24 hours.

In a similar manner to that described in the above-mentioned article, the supernatant of the medium was used for purifying the viruses. The purified viruses were inactivated by heating for one hour at a temperature of 56° C. and were used as an antigen for the primary immunization.

A fraction of glycoprotein of the viruses prepared in the following manner was used as a booster dose for intensifying the immunization:

The H9/HTLV-III<sub>B</sub> cells cultured by the above-mentioned method were washed three times with PBS and were then centrifuged (2000 r.p.m./5 min) to obtain cell pellets. The cells ( $2 \times 10^8$ ) were washed three times with PBS saline (0.015M); (pH 7.2). The cells were solubilized by adding a cell lysing buffer solution [prepared by omitting sodium deoxycholic acid from RIPA buffer solution (5 ml) containing 1% Triton-X, 0.5% sodium salt of deoxycholic acid, 0.1% SDS, 0.15M NaCl and 0.05M Tris-HCl and having a pH of 7.2] and incubated at a temperature of 4° C. for 60 minutes. The lysate was centrifuged (3000 r.p.m./10 min). The supernatant was collected and heated at a temperature of 56° C. for one hour. The resultant solution was added to FCS-Sepharose [prepared by binding fetal calf serum (20 mg/ml) to Sepharose 4B (1 ml)] and reacted at a temperature of 4° C. overnight (for about 12 hours). The reaction solution was centrifuged (3000 r.p.m./10 min) to obtain a supernatant which was then used as the test sample.

The sample solution (1 ml) was added to ConA-Sepharose (0.5 ml; commercial product of Sigma) and was incubated at a temperature of 4° C. overnight (for about 18 hours). The material was put into a column and, after washing with PBS, elution was effected using  $\alpha$ -methyl-D-glucoside (0.5M; 3 ml). The effluent was collected and divided into small fractions (each 0.5 ml).

Sera were collected from hemophiliac patients who were the healthy carriers of HIV. From the collected sera one exhibiting the highest antibody titre against the envelope was selected by the Western blotting method and purified to obtain IgG fraction. Each lysate was added to Sepharose 4B bound with the purified IgG (5 mg/ml) [hereinafter referred to as anti-HIV-Sepharose] and was incubated at a temperature of 4° C. for more than 4 hours. The anti-HIV-Sepharose was put in a column, washed with PBS and eluted with 0.2M



glycine-buffered solution (pH 2.7). The eluent containing 0.1 mg/ml of the antigen was used as a booster for intensifying the immunization.

## 2) Preparation of hybridoma:

Purified viruses were inactivated by heating at a temperature of 56° C. for one hour. The viruses (0.1 ml) were mixed with Freund's complete adjuvant (0.1 ml) and used for primary immunization of a Balb/c mouse (purchased from Kuroda Dobutsu K.K., Japan). Then a purified antigen solution of virus glycoprotein (each 0.1 ml) mixed with Freund's incomplete adjuvant (each 0.1 ml) was used as a booster dose and was intraperitoneally given to the animal 3 times at intervals of 2 weeks. 3 days after the final immunization, the spleen cells were collected from the mouse in conventional manner. The spleen cells were mixed with P3-X63-Ag8 (X63) [Nature, 256, 495-497 (1975)], the ratio of the number of spleen cells to the number of myeloma cells being 1:5. The mixture was centrifuged (1200 r.p.m./5 min), followed by removal of the supernatant. The pelleted cells were well loosened and a mixed solution (0.2-1 ml/10<sup>3</sup> of antibody-producing cells) of polyethyleneglycol 4000 (PEG-4000; 2 g), MEM (2 ml) and dimethylsulfoxide (0.7 ml) was added to the antibody-producing cells with stirring. After this, MEM (1-2 ml at a time) was added several times to the mixture at intervals of 1-2 minutes, followed by addition of MEM to make up to 50 ml in total. The cell suspension was centrifuged to remove the supernatant. The cell pellets were loosened and a normal medium [100 ml: prepared by adding 10% FCS to RPMI-1640] was added thereto. The cells were loosened by gentle pipetting.

The cell suspension was poured into each well of 24-well culture plates in an amount of 1 ml per well, followed by incubating at a temperature of 37° C. for 24 hours using a CO<sub>2</sub> incubator. After adding to the culture plate a HAT medium [prepared by adding to a normal medium, hypoxanthine (10<sup>-6</sup>M), thymidine (1.5×10<sup>-5</sup>M) and aminopterin (4×10<sup>-7</sup> M)], the culturing was effected further for 24 hours. For 2 days after this, the supernatant (1 ml) was removed, and the same amount of fresh HT medium was added to each well at an interval of 24 hours. The culturing was effected further for 10-14 days at a temperature of 37° C. using an CO<sub>2</sub> incubator.

When the presence of fused cells (about 300) grown in the form of colonies in certain wells was found, on each occasion, the supernatant (1 ml) was removed from the well and replaced by fresh HT medium (1 ml: prepared by omission of aminopterin from HAT medium). Such a replacement by HT medium was effected further for 2 days at an interval of 24 hours.

After culturing for 3-4 days using HT medium, part of the supernatant was collected from each of the above-mentioned cultures for assaying the ability to bind to the surfaces of H9 cells infected with HTLV-III<sub>B</sub> by the above-mentioned fluorescein antibody method. A clone exhibiting the highest binding activity was designated as 54<sup>C</sup>C which was further subjected to subcloning to select a subclone designated as 54<sup>C</sup>CB1 which grew vigorously to exhibit the highest productivity of the antibody.

## 3) Preparation of monoclonal antibodies by the use of 54<sup>C</sup>CB1:

Hybridoma cells of 54<sup>C</sup>CB1 prepared by method (2) were abnormally given to Balb/c mice [pristane-treated; 8 weeks old] in an amount of 4×10<sup>6</sup> cells/mouse. 10-21 days after this, ascites tumour was induced by the hybridoma cells. From the host mice of the ascites tumour, ascitic fluid was collected in an amount of 5-10 ml/mouse. After removal of solids from the ascites by centrifugation (3000 r.p.m./5 min),

the salting-out of the supernatant was effected using ammonium sulfate (40%). The solution was dialyzed against 0.04M phosphate-buffered solution containing NaCl (0.03M) and having a pH of 8.0. The residue was passed through a column packed with DE52 (bed volume 50 ml; commercial product of Whatman) at a flow rate of 20-30 ml per hour to collect IgG fractions which were used as purified monoclonal antibody (designated as 0.5β antibody).

## 4) Surface reactivity of 0.5β antibody:

The reactivity of the resultant antibody with the surfaces of H9 or CEM cells infected with HTLV-III<sub>B</sub> or LAV-1 was determined by the above-mentioned fluorescein antibody method to obtain results as shown in FIG. 1. The present antibody is reactive with the surfaces of H9 or CEM cells infected with HTLV-III<sub>B</sub> or LAV-1, but is not reactive with uninfected cells.

## 5) Binding characteristics of 0.5β antibody:

The antibody of the present invention was assayed by the Western blotting method (see FIG. 2A), the radioimmuno-precipitation method (see FIG. 2B), the cross-precipitation method (see FIGS. 2C and 2D) and by the digest pattern obtained using endoglycosidase H (see FIG. 2E). As a result, it has been found that the present antibody is capable of specifically binding to gp120 of the envelope protein located at the HIV envelope.

## A. Western blotting method:

With reference to Towbin, H., Stachelin, T. and Gordon, J. [Proc. Natl. Acad. Sci. USA 76, 4350-4354 (1979)], the Western blotting method was carried out in the following manner:

Purified HTLV-III<sub>B</sub> viruses [prepared by the method disclosed in Science 224, 497-500 (1984)] were subjected to electrophoresis using 12% SDS-PAGE [disclosed in Nature 227, 680-685 (1970)]. The gel was put onto a nitrocellulose membrane to transfer the viruses onto the surface of the membrane. The membrane was cut into strips having a width of 0.4-0.6 cm. On each occasion, the strips were put into one of the following antibody solutions (a)-(f) for incubation at room temperature for 4 hours:

(a) serum obtained from a hemophiliac patient having a positive activity against anti-HIV antibody;

(b) human serum (control);

(c) 0.5β antibody;

(d) VAK5 (anti-p24) monoclonal antibody produced by the hybridoma obtained by immunizing mouse with p24, a gag antigen of HTLV-III<sub>B</sub>, which is capable of recognizing p24 and its precursor [Jpn. J. Cancer Res. (Gann) 78, 235-241 (1987)];

(e) 52E5 (anti-p17) monoclonal antibody [monoclonal antibody produced by the hybridoma obtained by immunizing HTLV-III<sub>B</sub>, which is capable of recognizing p17 and its precursor, said p17 being one of the gag proteins of HTLV-III]; and

(f) mouse IgG<sub>1</sub> antibody (MOPC21) as control [Litton Biogenetics Inc. Catalogue No. 8401-03].

Samples (a) and (b) were diluted with PBS-BSA-Az (1:50) and Samples (d)-(f) were diluted with ascitic fluid (1:500) and treated in the following manner at room temperature unless otherwise specified.

The incubated strips were washed three times with PBS and incubated for 2 hours in a diluted solution (1:750) of biotin-conjugated anti-human or anti-mouse IgG antiserum (commercial product of TAGO). The strips were washed three times with PBS and immersed in a diluted solution (1:1000) of avidin conjugated with horseradish-peroxidase (commercial product of Sigma) for incubation for one hour. After this, the material was washed three times with PBS

and treated with a colour-developing reagent containing 4-chloro-1-naphthol (commercial product of BioRad). The results are shown in FIG. 2A, in which (a)-(f) correspond respectively to the above mentioned antibodies. FIG. 2A indicates that 0.5  $\beta$  antibody is capable of recognizing the envelope of HTLV-III<sub>B</sub> having a molecular weight of 120 Kd.

#### B. Radioimmunoprecipitation method:

H9/HTLV-III<sub>B</sub> ( $2 \times 10^7$  cells) was labelled with  $^{35}\text{S}$  cysteine by incubating for 4 hours in a solution containing  $^{35}\text{S}$  cysteine (100  $\mu\text{Ci/ml}$ ). After washing with PBS, the cells were put into RIPA buffer solution (0.5 ml). Soluble cell lysate was prepared by breaking the cell membrane using a vortex, followed by centrifugation (10000 r.p.m./one hour).

The lysate was subjected to reaction with normal human IgG (500  $\mu\text{g}$ ) [purified using Protein A Sepharose, with reference to a guidebook of affinity-chromatography (Principles & Methods, p48-52, published by Pharmacia Fine Chemicals AB.)] for 18 hours. The reaction solution as centrifuged (10000 r.p.m./10 min) to collect the supernatant which was used as a sample solution. The sample solution (40  $\mu\text{l}$ ) was mixed with immuno-beads [prepared by binding, on each occasion, 40  $\mu\text{g}$  of one of the following antibodies (a)-(e) with 20  $\mu\text{l}$  of Sepharose 4B], incubated at a temperature of 4° C. for 4 hours.

- (a) serum obtained from a patient having a positive activity against anti-HIV antibody (control);
- (b) human serum (control);
- (c) 0.5 $\beta$  antibody;
- (d) VAK5 (anti-p24) (control);
- (e) MOPC21 (control).

After washing 4 times with PBS, the beads were suspended in a sample buffer (50  $\mu\text{l}$ ) and heated for 2 minutes under reflux to elute the bound protein. On each occasion, the supernatant was subjected to electrophoresis using 12% SDS-PAGE and assayed by autoradiography in conventional manner. The results shown in FIG. 2B indicate that 0.5 $\beta$  antibody is capable of recognizing both gp120 which is a mature protein and gp160 which is a precursor of gp120.

In this case, a mixture of 0.75M tris-HCl buffered solution (8.3 ml; pH 6.8), 10% SDS (20 ml), glycerol (10 ml), 2-mercaptoethanol (5.0 ml), H<sub>2</sub>O (6.7 ml) and bromophenol blue (1 mg) was used as a sample buffer solution (X2) [Laemmli, U.K., Nature, 227, 680-685 (1970)].

#### C. Cross-precipitation method:

HTLV-III<sub>B</sub> was purified by the sucrose density-gradient ultracentrifugation to collect the active fraction having a specific gravity of about 1.16. The viruses were inactivated by heating at a temperature of 56° C. for one hour, followed by disruption with 0.5% NP40 to obtain purified viruses. The purified viruses (10  $\mu\text{l}$ ) were put in RIPA buffer solution (40  $\mu\text{l}$ ) and incubated together with Sepharose 4B bound to 0.5 $\beta$  antibody or Sepharose 4B bound to MOPC21 (prepared in a similar manner to that described above) at a temperature of 4° C. overnight (for about 18 hours). The reaction solution was centrifuged (5000 r.p.m./5 min) to give a supernatant, from which the following test samples were prepared:

- (b) supernatant pre-treated with 0.5 $\beta$  Sepharose;
- (c) supernatant pre-treated with MOPC21-Sepharose used as control; and
- (a) untreated.

On each occasion, the sample was subjected to electrophoresis using 12% SDS-PAGE and blotted onto a nitrocellulose membrane. The membrane was subjected to reaction with the serum of a healthy host of HIV (diluted  $\times 100$ ). The colour of the reaction product was developed by the method of 2-A using biotin-avidin-peroxidase. It has been observed

that by treating with 0.5 $\beta$  antibody, the reactivity of envelope gp120 with the serum of the patient decreased, while the reactivity of control samples was not significantly changed. With regard to the reactivity with other proteins, no difference was found between the sample treated with 0.5  $\beta$  antibody and the control sample.

#### D. Cross-precipitation method:

A similar procedure to that described in C as above was carried out using a lysate of H9/HTLV-III<sub>B</sub> labelled with  $^{35}\text{S}$  cysteine instead of purified viruses in the following manner:

Part of lysate supernatant of H9/HTLV-III<sub>B</sub> labelled with  $^{35}\text{S}$  cysteine was incubated together with Sepharose 4B bound to 0.5  $\beta$  antibody or Sepharose 4B bound to MOPC21 prepared in a similar manner to that described above at a temperature of 4° C. overnight (for about 18 hours). The mixture was centrifuged (5000 r.p.m./5 min) to obtain the supernatant, which was designated as a sample treated once. Similarly, this sample was incubated for 4 hours and centrifuged to obtain a test sample treated twice.

On each occasion, the supernatant of part of the sample of lysate treated once or twice or the supernatant of part of untreated sample was subjected to reaction with an anti-HIV-IgG Sepharose [Sepharose bound to IgG having anti-HIV antibody activity] at a temperature of 4° C. for 4 hours. The reaction mixture was washed with a RIPA-buffered solution 4 times, eluted using a sample buffer solution, treated with 12% SDS-PAGE and assayed by conventional autoradiography in a similar manner to that described in 2-B as described hereinbefore.

The results are shown in FIG. 2D, which indicates as follows:

- (b) When Sepharose 4B bound with 0.5 $\beta$  antibody cleared the sample once, gp120 was significantly decreased; and
- (c) when cleared twice, both gp120 and gp160 almost disappeared;
- (d) On the other hand, when the control IgG<sub>1</sub> cleared the lysate twice, a decrease of both gp120 and gp160 was not noted.

The result from untreated sample (control) is also shown in (a).

#### E. Digest pattern by using endoglycosidase:

0.5% NP40 was added to purified HTLV-III<sub>B</sub> (10  $\mu\text{l}$ ). The mixture was incubated at a temperature of 4° C. for 24 hours to disrupt the cells, followed by dialysis against 0.1M sodium citrate (pH 5.5). Endoglycosidase H (commercial product of NEN; 0.25 U) was added to the disrupted viruses which were then incubated at a temperature of 37° C. for 3 hours to digest the viruses.

On each occasion, one sample selected from (b) digested HTLV-III<sub>B</sub> and (a) HTLV-III<sub>B</sub> treated with buffered solution was subjected to electrophoresis using 12% SDS-PAGE and blotted onto a nitrocellulose membrane. In a similar manner to that described in A hereinbefore, colour was developed by using 0.5 $\beta$  antibody to develop colour on the strips of the nitrocellulose membrane. The results are shown in FIG. 2E, which indicates that 0.5 $\beta$  antibody is also reactive with protein portion (70-84 Kb), from which sugar chains were cut off by endoglycosidase.

6) Inhibiting activity of 0.5 $\beta$  antibody against the formation of syncytia by the action of HTLV-III<sub>B</sub>:

H9/HTLV-III<sub>B</sub> ( $2.5 \times 10^4$  cells) was incubated together with 50  $\mu\text{g/ml}$  of MCOP 21 ascites (FIGS. 3C, 3H), 50  $\mu\text{g/ml}$  of 0.5 $\beta$  ascites (FIGS. 3D, 3I) or 5  $\mu\text{g/ml}$  of 0.5 $\beta$  ascites (FIGS. 3E, 3J) at room temperature for 30 minutes in RPMI-1640 medium (100  $\mu\text{l}$ ) containing 10% FCS, to which was then added  $5 \times 10^4$  CEM cells (50  $\mu\text{l}$ ) for culturing at a

temperature of 37° C. for 18 hours using an incubator containing 5% CO<sub>2</sub>. Similarly, each of CEM (FIGS. 3A, 3F) and H9/HTLV-III<sub>B</sub> (FIGS. 3B, 3G) was cultured and used as control. After culturing, an inverted microscope was used to take photographs. FIGS. 3A-E (X 150) and FIGS. 3F-J (X 60) were taken after culturing for 18 hours and a further culturing for 3 days respectively. It was found that the formation of syncytia (FIG. 3C) and the formation of cell clumps (FIG. 3H) in the control samples were completely inhibited in the presence of 50 µg/ml of 0.5β antibody (FIGS. 3D, 3I). The formation of smaller numbers of syncytia (FIG. 3E) and the formation of smaller cell clumps (FIG. 3H) were observed in the presence of diluted 0.5β antibody (5 µg/ml). In this case, it was observed that the cells did not form larger clumps and scattered around the syncytia and the cell clumps. From this fact, it appears that the use of 50 µg/ml of 0.5β antibody effectively inhibits infection between the cells, while the use of 5 µg/ml of 0.5β antibody gave partial inhibition. On each occasion, the ascites was inactivated at a temperature of 56° C. for one hour before use.

#### 7) Neutralizing activity of 0.5β antibody:

In a similar manner to that disclosed in JP-A-500767/86 and JP-A-28756/86, HTLV-III<sub>B</sub> viruses (2-5×10<sup>8</sup>/ml) were collected by ultracentrifugation (32,000×g, 3 hours) of the supernatant of a culture of H9/HTLV-III<sub>B</sub>. On each occasion, the viruses (20 µl) and 0.5β antibody (20 µl) at different dilution ratios were put into each well of a 96-well round bottom culture plate for incubation at a temperature of 4° C. for one hour, followed by further incubation at room temperature for 15 minutes.

H9 cells (4×10<sup>4</sup>) were treated at room temperature for 20 minutes using polyburen (2 µg/ml), and washed with RPMI-1640 medium. Then the cells were transferred to another RPMI-1640 medium (200 µl) containing 20% FCS, penicillin (50 U/ml) and streptomycin (50 µg/ml) for incubation at a temperature of 37° C. for 5-6 days.

The presence of HTLV-III<sub>B</sub> p24 antibody in H9 cells was assayed by the fluorescein antibody method to calculate the titre of the neutralizing antibody [Nature 316, 72-74 (1985)].

The results are shown in Table 1. In this table, the neutralizing activity of the antibodies is expressed by ng/ml of purified IgG or by the reciprocal number of the dilution ratio of the serum and ascites.

TABLE I

Neutralizing antibody titre		
Purified 0.5β	0.5β in ascites	Human serum (positive)
100 ng/ml	>6250	280

0.5β antibody of the present invention exhibits a neutralizing activity at low concentration such as 100 ng/ml of purified antibody. In the form of ascites, a 100% neutralizing activity was observed at a dilution ratio of 1:6250.

#### 8) Purification of envelope antigen from H9/HTLV-III<sub>B</sub> cells:

H9/HTLV-III<sub>B</sub> was cultured and treated to obtain a pellet in a similar manner to that described in (1) hereinbefore. The pellet (5×10<sup>7</sup> cells) was suspended in RIPA buffer (2.5 ml)

and incubated at a temperature of 4° C. for 60 minutes to obtain a cell lysate. The cell lysate was centrifuged (3000 r.p.m./10 min) to remove solids. The supernatant was heated at a temperature of 56° C. for one hour to inactivate HTLV-III<sub>B</sub>. The inactivated cells were subjected to reaction with about 0.5 ml of FCS-beads [Sephacrose 4B bound to 10% FCS] at a temperature of 4° C. for 18 hours and centrifuged (5000 r.p.m./5 min).

Non-specific adsorbates were adsorbed onto the FCS-beads.

The supernatant was mixed with 0.5β antibody-bound Sepharose 4B beads (200 µl) and incubated at a temperature of 4° C. for 4 hours. After completion of the reaction, the beads were packed into a column and washed with 4 ml of PBS (pH 7.2) and 2 ml of HSB [0.02M phosphate-buffered solution containing 0.5M NaCl; pH 7.2]. Elution was effected using glycine-HCl (4 ml; pH 2.7) containing 0.15M NaCl. Immediately after this, the eluent was neutralized with Tris-HCl (pH 10). From each fraction (0.5 ml), 10 µl was collected and smeared into 2 wells of a ELISA plate Imuron I (commercial product of Dyantech Laboratories, U.S.A.). The sample was incubated at a temperature of 4° C. overnight in 0.09 ml of 0.1M carboxylic acid buffered solution (pH 9.8).

The ELISA method was carried out overnight to detect the eluted fraction, by which reaction with 0.5β antibody (X1500) was effected, followed by reaction with alkaline phosphatase-conjugated anti-mouse IgG to develop colour. In FIG. 4 purified antigen is shown by a single peak.

I claim:

1. A monoclonal antibody or fragment thereof having the following characteristics:

- which specifically binds to a glycoprotein antigen having a molecular weight of 120,000 daltons and located in the envelope of HTLV-III (HIV-I) virus;
- which specifically neutralizes
  - infection by cell-free virions in vitro and
  - cell-to-cell infection of said virus in vitro by being capable of binding to the surface of a cell infected with HTLV-III (HIV-I) virus thereby to inhibit the formation of syncytia induced from infected cells and uninfected cells;
- which specifically binds to a precursor of a glycoprotein antigen, said precursor having a molecular weight of 160,000 daltons;
- which specifically binds to an epitope located within amino acid sequence of 308 to 331 of HTLV-III (HIV-1) gp160;
- which is classified as IgG<sub>1</sub>; and
- wherein the antibody is the 0.5β monoclonal antibody produced by the 54/CBI hybridoma (ECACC 87051401).

2. Hybridoma 54/CBI, ECACC No. 87051401.

3. A composition, which comprises as active ingredient a monoclonal antibody or a fragment thereof as claimed in claim 1, together with at least one member selected from the group consisting of a pharmaceutically acceptable carrier and an excipient.

\* \* \* \* \*



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**United States Patent** [19]

Ohno

[11] Patent Number: **5,169,752**[45] Date of Patent: **Dec. 8, 1992****[54] METHODS AND MATERIALS FOR HIV DETECTION**[75] Inventor: **Tsuneya Ohno, Ridgewood, N.J.**[73] Assignee: **Nissin Shokuhin Kabushiki Kaisha, Osaka, Japan**[21] Appl. No.: **668,386**[22] Filed: **Mar. 13, 1991****Related U.S. Application Data**

[60] Continuation of Ser. No. 340,702, Apr. 20, 1989, abandoned, which is a division of Ser. No. 146,371, Feb. 3, 1988, abandoned, which is a continuation-in-part of Ser. No. 64,066, Jun. 29, 1987, abandoned, which is a continuation-in-part of Ser. No. 16,282, Feb. 19, 1987, abandoned.

[51] Int. Cl.<sup>5</sup> ..... **C12Q 1/70; C12Q 1/00; G01N 33/53**

[52] U.S. Cl. .... **435/5; 436/548; 436/813; 435/974**

[58] Field of Search ..... **435/5, 7; 436/548, 813**

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Murray & Bicknell

[57]

**ABSTRACT**

Disclosed are immunologically active polypeptides, preferably antibodies or antibody fragments, and most preferably monoclonal antibodies, which are reactive with idiotypes of antibodies to human lymphocyte T4 protein and are reactive with the HIV virion in a manner allowing for in vitro and in vivo neutralization of

HIV infectivity and detection of HIV particles in biological fluids. Presently preferred embodiments comprise monoclonal anti-monoclonal-anti-human lymphocyte T4 anti-bodies produced by new murine hybridoma cell lines JT4C8, JT4C12, JT4C16, JT1-1F3, JT1-1F3-E5, JT1-1D7 and JT2-N15. Also disclosed are active and passive vaccination procedures.

**7 Claims, 6 Drawing Sheets**

FIG. 1

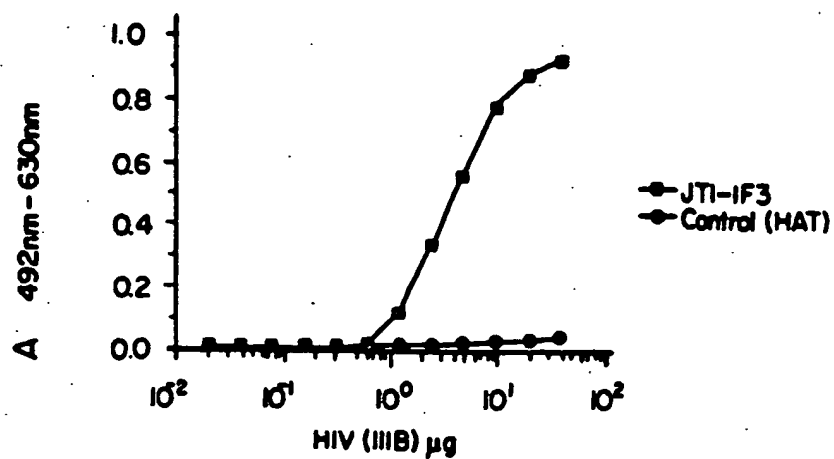


FIG. 2

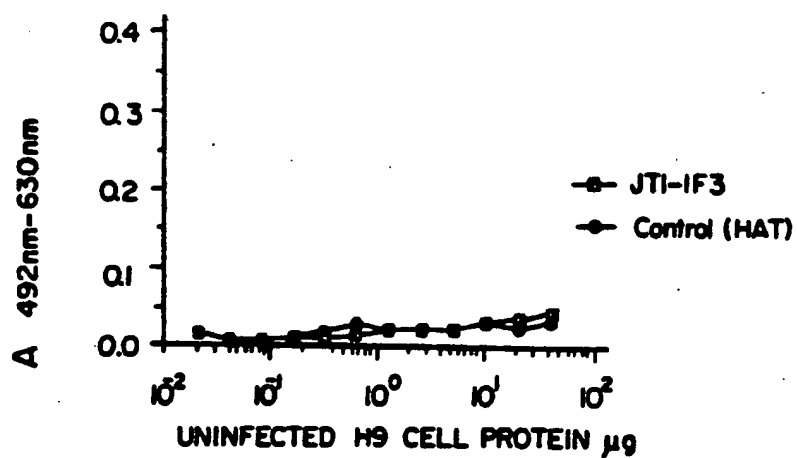
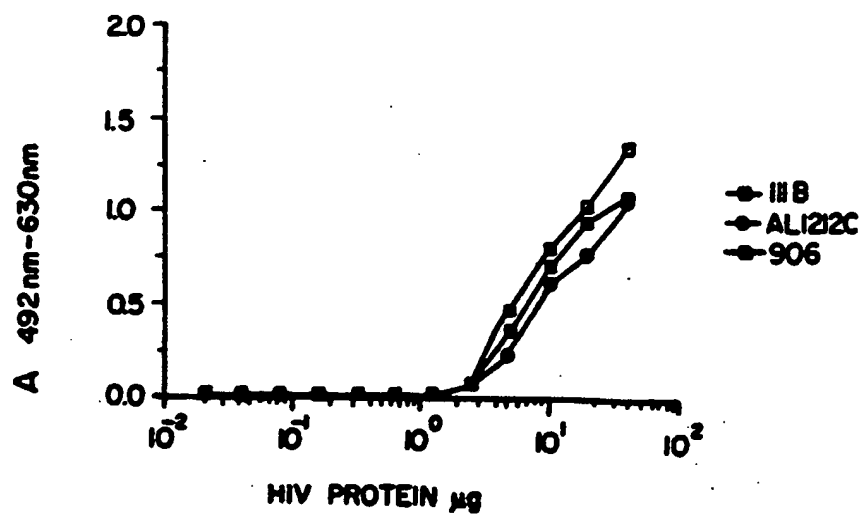


FIG. 3



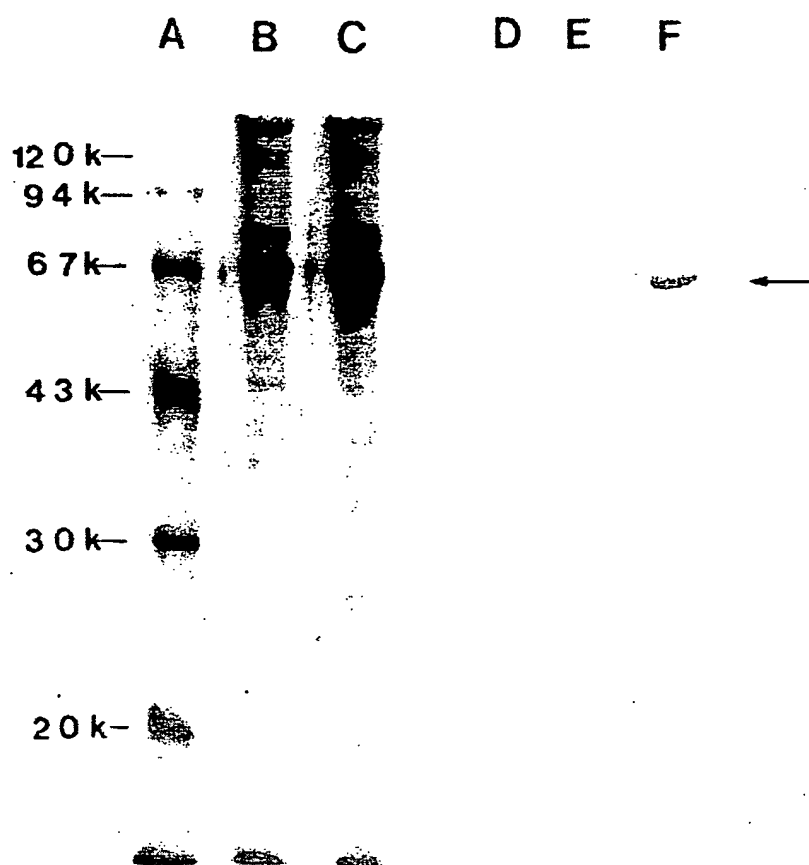


FIG. 4



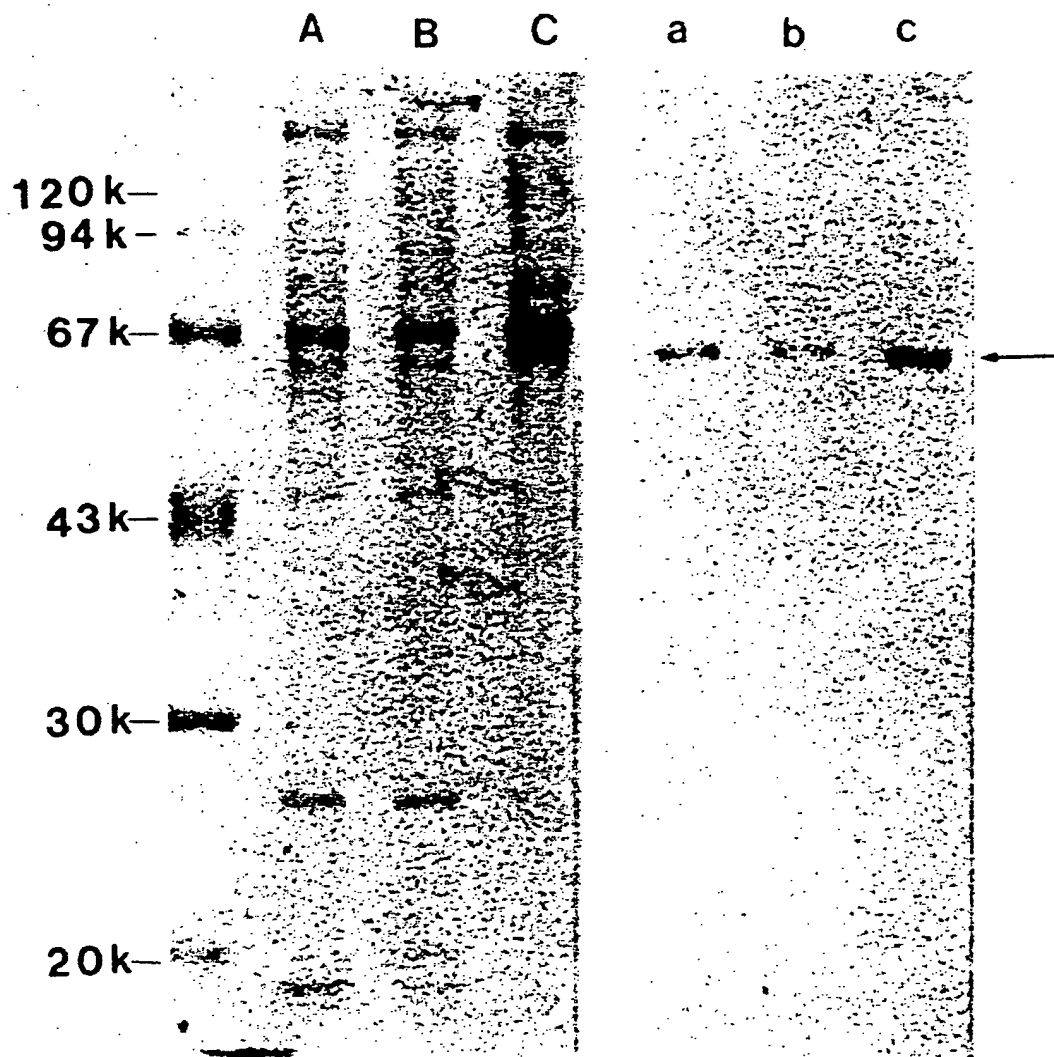


FIG. 5

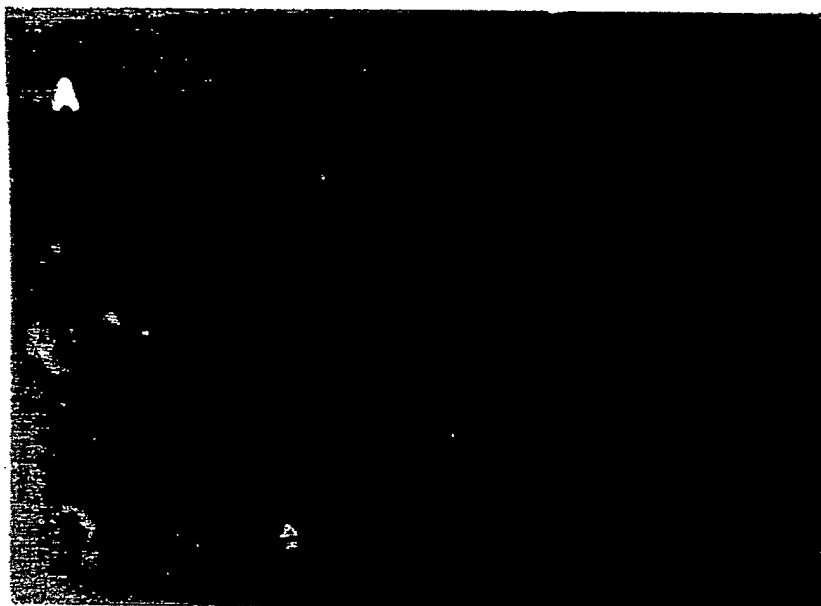


FIG.6A



FIG.6B

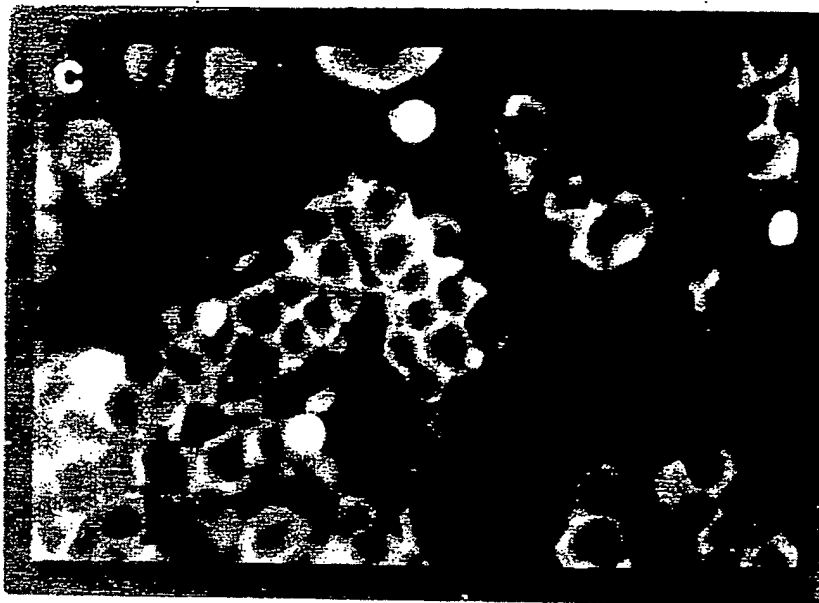


FIG. 6C

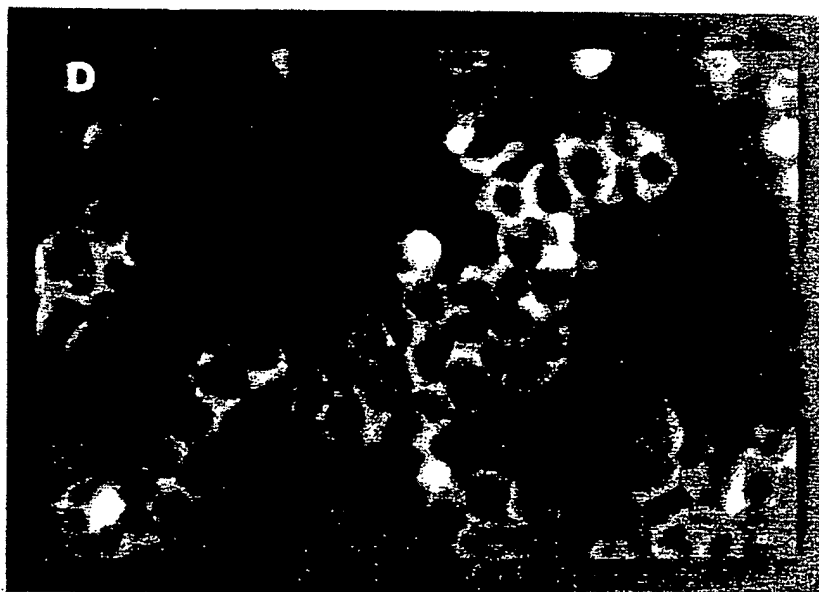


FIG. 6D

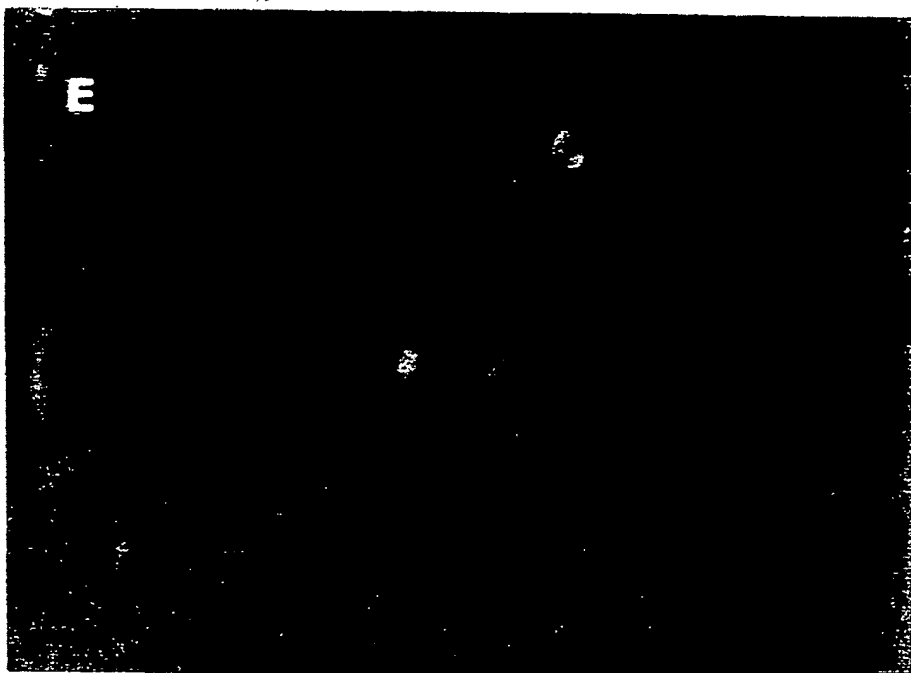


FIG. 6E

## METHODS AND MATERIALS FOR HIV DETECTION

This application is a continuation of application Ser. No. 07/340,702, filed Apr. 20, 1989 now abandoned, which is a division of 07/146,371 filed Feb. 3, 1988, now abandoned, which is a continuation-in-part of co-pending U.S. patent application Ser. No. 064,066, filed Jun. 29, 1987, which is in turn a continuation-in-part of U.S. patent application Ser. No. 016,282, filed Feb. 19, 1987.

### BACKGROUND

The present invention relates generally to methods and materials useful in the diagnosis and treatment of infection with Human Immunodeficiency Virus (HIV). More particularly, the invention relates to immunologically active polypeptides, preferably antibodies or antibody fragments, and most preferably monoclonal antibodies, which are reactive with idiotypes of antibodies to human lymphocyte T4 protein and also reactive with the HIV virion in a manner allowing for *in vitro* and *in vivo* neutralization of HIV infectivity. Moreover, the invention relates to immunologically active polypeptides useful in vaccine compositions for developing protective responses to HIV infection.

The state of the art with respect to the epidemiology and immunology of the causative agent of AIDS in humans is well summarized in: Laurence, "The Immune System in AIDS", *Scientific American*, December, 1985, pp. 84-93; Gallo, "The First Human Retrovirus", *Scientific American*, December, 1986, pp. 88-98; Gallo, "The AIDS Virus", *Scientific American*, January, 1987, pp. 47-56; Levy et al., *Science*, 225, 840-842 (1984); "Mobilizing Against AIDS", Institute of Medicine, National Academy of Sciences, Harvard University Press (Cambridge, Mass. 1986); and, Lane et al., *Ann. Rev. Immunol.*, 3, pp. 477-500 (1985). The role of T4 surface glycoprotein (sometimes referred to as "CD4" protein or determinant) of human T lymphocytes in infection by HIV has been extensively studied as represented by Dalgleish et al., *Nature*, 312, pp. 763-767 (1984); Klatzmann et al., *Science*, 312, 767-768 (1984); Klatzmann et al., *Science*, 225, pp. 59-62 (1984); McDougal et al., *J. Immunol.*, 135, pp. 3151-3162 (1985); and, Maddon et al., *Cell*, 47, pp. 333-348 (1986). See also, Marrack et al., "The T Cell and Its Receptor", *Scientific American*, February 1986, pp. 36-45; and, McDougal et al., *Science*, 231, 382-385 (1986).

It has recently been projected that soluble forms of CD4 may have therapeutic utility in treatment of HIV infection. See, Fisher et al., *Nature*, 331, 76-77 (1988); Hussey et al., *Ibid.*, at pp. 78-81; Deen et al., *Ibid.*, at pp. 82-83; and Traunacker et al., *Ibid.*, at pp. 84-86 all of which relate to *in vitro* neutralization of HIV infectivity by soluble CD4. Among the potential drawbacks to the projected use of soluble CD4 therapeutic agents is the known reactivity of CD4 with class II major histocompatibility complex ("MHC") molecules present on the surface of other immune cells including B cells, macrophages and monocytes, leading to the suggestion that CD4 may need to be modified (e.g., by truncation) prior to attempted therapeutic use.

Numerous reports appear in the literature relating to the potential of antibodies to neutralize infectivity of HIV *in vitro* and *in vivo* and specifically to attempts at active immunization for the purpose of developing protective immunity. See, e.g., Matthews et al., *Proc. Nat'l*

*Acad. Sci. (USA)*, 83, pp. 9709-9713 (1986); Norman, "AIDS Therapy: A New Push For Clinical Trials", *Science*, 230, pp. 1355-1358 (1985) and prior articles in this series; Newmark, *Nature*, 324, pp. 304-305 (1986); and notes appearing in *Scientific American*, February, 1987, at pages 86-88 under the heading, "AIDS: Hope . . . And Warnings", and in *New Scientist*, Dec. 18, 1986, page 7, under the heading "Can Protein T Thwart The AIDS Virus . . .?". See also, Mitsuya et al., *Nature*, 325, 773-778 (1987); Kennedy et al., *Science*, 231, 1556-1559 (1986); Chanh et al., *EMBO Journal*, 5(11), 3065-3071 (1986); Chanh et al., *Eur. J. Immunol.*, 16, 1465-1468 (1986); Putney et al., *Science*, 234, 1392-1395 (1986); and, Matshushita et al., Abstract W.3.2, p.106, "III International Conference on Acquired Immunodeficiency Syndrome (AIDS)", Jun. 1-5, 1987.

Of interest to the background of the present invention are the published results of investigation into the immunological role of anti-idiotypes. See, e.g., Kennedy et al., "Anti-Idiotypes and Immunity", *Scientific American*, July, 1986, pp. 48-56; Jerne, "The Immune System", *Scientific American*, July, 1973, pp. 52-60; Marx, "Making Antibodies Without The Antigens", *Science*, 228, pp. 162-165 (1985); Finberg et al., *CRC Critical Reviews in Immunology*, 7, 269-284 (1987); and Kennedy et al., *Science*, 232, pp. 220-223 (1986). See, also, Norman, *supra*, relating to a potential correlation between anti-HIV-immunotherapy and production of anti-idiotypic antibodies to the HIV surface proteins.

Of particular interest to the background of the present invention is the work reported by McDougal et al., *J. Immunology*, 137, 2937-2944 (1986) wherein it was noted that: "... rabbit anti-idiotypic sera raised against each of four candidate CD4 monoclonal antibodies [OKT4A, OKT4D, OKT4F and Leu3a (sometimes referred to as "anti-Leu3a")] did not react with [HIV] virus or inhibit virus binding to CD4+ T cells." This notation should be compared with the recent oral presentations of Ronald C. Kennedy at the 7th Annual DNA/Hybridoma Congress, San Francisco, Mar. 1-4, 1987, as reported in *Bio/Technology*, 5, 421-422 (1987), and at the III International Conference on Acquired Immunodeficiency Syndrome (AIDS), Washington D.C. Jun. 1-5, 1987, (see, Abstract TH.9.5) as reported in *New Scientist* Jun. 11, 1987 at page 26. In these presentations the development of polyclonal antisera against anti-T4 antibodies was noted, as was the capacity of such antisera to recognize HIV and partially neutralize HIV infectivity *in vitro*. The latter presentation also mentioned preparation of monoclonal anti-idiotypic antibody and this development is also described in Chanh et al., *Proc. Nat'l Acad. Sci. (USA)*, 84, 3891-3895 (June, 1987).

There continues to exist a substantial need in the art for new methods and materials for diagnosis for the presence of HIV particles and HIV-infected cells in biological fluid and tissue specimens and also a substantial need for new means for effecting *in vivo* neutralization of the infectivity of HIV and the development of vaccination procedures conferring immunological protection against HIV infection.

### BRIEF SUMMARY

The present invention provides purified and isolated immunologically active polypeptides, preferably antibodies or chimeric antibodies or fragments thereof, and most preferably monoclonal antibodies, which are reactive with idiotypes of antibodies (preferably mono-

clonal antibodies) to human lymphocyte T4 protein. Such products of the invention are capable of specific immunobinding with that portion of the HIV virion which is necessarily interactive with T4 surface proteins during infection by HIV of host cells such as human T lymphocytes and cells of the human nervous system. These products of the invention may be characterized, *inter alia*, by their strain independent capacity to neutralize infectivity of HIV *in vitro*, by their specific reactivity with HIV protein having a molecular weight of from about 60,000 to about 80,000 as determined by SDS-PAGE, and by their non-reactivity with class II major histocompatibility molecules associated with human immune cell surfaces.

In one of its aspects, therefore, immunologically active products are generated which "respond" through specific immunobinding to HIV particles and to the surfaces of HIV-infected cells, even though they are generated without direct immunological reference to such surface proteins.

Purified and isolated polypeptides according to the invention are conspicuously suitable for use in assay procedures for the detection and/or quantification of HIV in biological fluids wherein the detection procedure is based upon immunobinding between HIV particles and reactive polypeptides (e.g., antibodies) of the invention. Moreover, antibodies, chimeric antibodies and fragments thereof developed according to the invention are selectively immunoreactive with the surfaces of HIV-infected cells and thus provide useful reagents for detection of HIV-infected cells in fluid and tissue samples and for segregation of HIV-infected cells from cell populations including both infected and non-infected cells. As such, products of the invention will be useful in diagnostic and therapeutic procedures involving separation and/or selective destruction of HIV-infected cells.

Purified and isolated immunologically active materials of the invention are also conspicuously suitable for use in anti-HIV treatment of animals, especially humans, susceptible to infection with HIV. According to one such method, immunologically effective amounts of, e.g., monoclonal antibodies of the invention, are administered to a patient either already infected with HIV or to a patient at risk of infection with HIV to develop passive immunity with respect to HIV infection. According to another method, cells infected with HIV are subjected to, e.g., *in vitro* segregation from non-infected patient cells and the latter may be returned to the patient.

As set forth in the following detailed description, antibody-related polypeptides of the present invention are preferably obtained by means of initial development of mono-specific antibodies (preferably monoclonal antibodies) to the human lymphocyte T4 glycoprotein (the CD4 determinant) followed by preparation of antibodies (preferably monoclonal antibodies) to the T4 idiotype region of antibodies formed in the initial development step.

Chimeric antibodies and fragments thereof and especially bi-specific antibodies are also products within the contemplation of the present invention, as are antibody-related products produced in microbial hosts, (e.g., procaryotic and eucaryotic cells in culture) which hosts are transformed or transfected with DNA sequences encoding the desired polypeptide products.

As one example, with structural information in hand concerning the idiotype regions of antibodies of the

invention, it becomes possible to employ procaryotic and eucaryotic hosts such as *E. coli*, yeast, insect, and mammalian cells in culture to produce useful antibody fragments (such as fab' and f(ab')<sub>2</sub> fragments). Moreover, it is within the contemplation of the invention that chimeric antibodies (e.g. mouse/human antibodies) may be prepared using transformed mouse myeloma cells or hybridoma cells (especially heavy chain deletion mutant cells) as production hosts. Hybridoma cell producing bi-specific antibodies having diagnostic and therapeutic uses are contemplated. Recombinant methods may also be applied to the production of HIV subunit vaccine materials. For example, monoclonal antibodies of the invention are expected to be extremely well-suited for the screening of expression products of HIV DNA in transformed or transfected vial or procaryotic hosts, allowing isolation of DNA encoding all or part of the amino acid sequence of naturally occurring immunologically significant HIV proteins (including glycoproteins). In suitable hosts, the presence of such DNA may allow for high level production of vaccine materials.

In a preferred form, the invention provides antibody-related polypeptides characterized as monoclonal anti-monoclonal-anti-human lymphocyte T4 antibodies. Especially preferred are monoclonal anti-OKT4 and anti-OKT4A antibodies, both of which are reactive with 60-80 Kd HIV proteins. Presently most preferred are monoclonal anti-OKT4A antibodies which have substantial capacity for *in vitro* neutralization of HIV infectivity of multiple HIV strains and are participative in complement-mediated cytolysis of HIV infected cells.

In another aspect, the present invention provides, for the first time, hybridoma cell lines which produce "anti-idiotypic" monoclonal antibodies specifically immunoreactive with a monoclonal antibody to human lymphocyte T4 protein in an antigen/antibody reaction. Illustratively, the present invention provides new murine-derived hybridoma cell lines, JT4C8, JT4C12 and JT4C16, JT1-1F3, JT1-1F3-E5, JT1-1D7 and JT2-N15, each of which produces, as a component of the supernatant of its growth in culture, a monoclonal antibody specifically reactive with anti-T4 idiotype and moreover reactive with HIV virion proteins in a manner expected to allow for both *in vitro* and *in vivo* neutralization of HIV infectivity.

Hybridoma cell line JT4C8 was received for deposit with the American Type Culture Collection, Rockville, Md., at the facilities of the U.S. Department of Agriculture, Plum Island, N.Y. on Feb. 18, 1987, with A.T.C.C. Accession No. HB9385. Hybridoma cell line JT4C12 was received for deposit with the American Type Culture Collection, Rockville, Md., at the facilities of the U.S. Department of Agriculture, Plum Island, N.Y. on Feb. 18, 1987, with A.T.C.C. Accession No. HB9387. Hybridoma cell line JT4C16 was received for deposit with the American Type Culture Collection, Rockville, Md., at the facilities of the U.S. Department of Agriculture, Plum Island, N.Y. on Feb. 18, 1987, with A.T.C.C. Accession No. HB9386. Hybridoma cell line JT1-1F3 was received for deposit at the European Collection of Animal Cell Cultures, Salisbury, Wiltshire, U.K. on Jun. 25, 1987, with ECACC Accession No. 87062501. Hybridoma cell line JT1-1F3-E5 was received for deposit at the European Collection of Animal Cell Cultures, Salisbury, Wiltshire, U.K. on Jun. 25, 1987 with ECACC Accession No. 87062502. Hybridoma cell line JT1-1D7 was received for deposit by the Fermentation

Research Institute, Ibaragi-ken, Japan on Jan. 29, 1988 with the Accession No. FERM BP-1685. Hybridoma cell line JT2-N15 was received for deposit by the Fermentation Research Institute, Ibaragi-ken, Japan on Jan. 29, 1988 with the Accession No. FERM BP-1684.

In still another of its aspects, the present invention provides for production of HIV subunit vaccine materials by means of well-known affinity purification methodologies whereby HIV protein fractions (especially those in the molecular weight range of from about 60,000 to about 80,000 and most especially about 65,000-67,000) are isolated through use of a selective immunoabsorbants prepared using antibodies of the invention.

Numerous aspects and advantages of the present invention will be apparent upon consideration of the illustrative examples and descriptions of practice of the invention in the following detailed description thereof and the drawing, wherein: FIGS. 1, 2 and 3 graphically represent the results of immunoreactivity testing of antibodies of the invention with HIV and noninfected cell proteins; FIGS. 4 and 5 provide immunoblot assay results involving antibodies of the invention and HIV proteins; and FIGS. 6A through 6E provide photographic results of immunofluorescent staining assays on infected and uninfected cells employing antibodies of the invention.

#### DETAILED DESCRIPTION

The following examples illustrate practice of the invention in the production of a number of hybridoma cell lines including JT4C8, JT4C12, JT4C16, JT1-1F3, JT1-1F3-E5, JT1-1D7 and JT2-N15 the isolation therefrom of monoclonal antibodies to anti-CD4, and the amplification and characterization of such monoclonal antibodies.

More particularly, Example 1 is directed to stimulation of a murine host toward production of antibodies to a commercially available anti-T4 monoclonal antibody, "OKT4", the fusion of spleen cells with myeloma cells, the screening, cloning and growth of hybridoma cells, and the isolation of monoclonal antibodies therefrom. Example 2 relates to the characterization of monoclonal antibodies so produced by fluorescent immunoassay, by Western blot assay for reactivity with HIV protein, and by screening for capacity to effect *in vitro* neutralization of HIV infectivity. Example 3 relates to a first procedure for development of hybridoma cell lines capable of providing in the medium of their growth monoclonal antibodies to the commercially available antibody "OKT4A". Example 4 relates to characterization of monoclonal antibodies so produced by means of immunofluorescence assay, Western blot assay, *in vitro* neutralization assay, ELISA assay, and fluorescence cell staining assays. Example 5 relates to a second procedure for development of hybridoma cell lines capable of providing in the medium of their growth monoclonal antibodies to the commercially available antibody "OKT4A" and to characterization of monoclonal antibodies so produced by means of Western blot assay, and *in vitro* neutralization assay.

#### EXAMPLE 1

According to one aspect of the practice of the invention, hybrid tumor cell lines are produced using standard immunological techniques such as described in Oi and Herzenberg, *Selected Methods Cell Immunology*, 351-372 (1979) and Godding, "Antibody Production

By Hybridomas", *J. Immunol. Meth.*, 39, pp. 285-308 (1980). Spleen cells from mice, hyperimmunized with monoclonal anti-T4 are fused with a mouse myeloma cell line in the presence of polyethylene glycol. The supernatant from growth of each "hybridoma" cell culture is tested for the presence of the desired antibody activity. Selected hybridoma cells are cloned to propagate cell lines which produce an antibody in their growth culture supernatant, which antibody has highly specific anti-anti-T4 activity.

#### A. Immunization

BALB/C mice each were subject to splenic injection with monoclonal anti-human lymphocyte T4 antibody (OKT4, Ortho Diagnostics, Rahway, N.J.).

One milligram of the lyophilized OKT4 material was brought up in 1 ml of distilled water. Dialysis was employed to remove and replace the original phosphate buffer with 50 mM MES buffer (Sigma Chemicals), pH 6.0. The material was then subjected to high pressure liquid chromatography separation on FPLC apparatus (Pharmacia, Laboratory Separation Division, Piscataway, N.J. 08854). Separation was carried out using a Mono Q column under recommended procedures except that the salt gradient was changed to 0 to 0.5M NaCl. Aliquots (20  $\mu$ l) of each 0.5 ml fraction were assayed for activity using freshly collected human lymphocytes ( $10^5$  cells per ml). More specifically, cells and HPLC fractions were mixed and centrifuged. The cells were then washed several times and resuspended in phosphate buffered saline (PBS). 5  $\mu$ g rabbit anti-mouse IgG labelled with FITC was incubated with the cells. Following centrifugation, the cell pellet was resuspended in PBS and results were read using a fluorometric cell counter. Fractions displaying highest activity were pooled, analyzed on SDS-PAGE and were revealed to be greater than 90% pure.

Each of 4 mice was initially given a splenic injection totalling approximately 100  $\mu$ l of inoculant (approximately 1.5  $\mu$ g OKT4 per mouse). Fourteen and twenty-eight days later the mice were each given booster injections of 100  $\mu$ l inoculant.

Four days after the final booster, the mice were sacrificed and spleens were removed aseptically and placed in petri dishes (on ice) containing Dulbecco's Modified Eagle's Medium (Gibco). The spleens were trimmed of fat and connective tissue, passed through 100 gauge stainless steel mesh. The resulting individual spleen cells were pelleted by centrifugation for 10 minutes at 1000 rpm. The cell pellet was washed twice with media (as above) and was resuspended in RPMI 1640 and the cell concentration was determined by counting in a hemocytometer in the presence of 0.2% trypan blue.

Mouse myeloma cells NS1/1.Ag4.1 derived from Balb/c strain, were grown in RPMI 1640 medium containing 15% heat-inactivated horse serum (Pel-Freeze). The cells were pelleted by centrifugation at 1000 rpm for 10 minutes and washed with RPMI 1640 containing no antibiotic. The cell concentration was determined by counting after resuspension in the same medium.

Spleen and NS1/1 cells were combined in a ratio of 4:1 and centrifuged at 1000 rpm for 10 minutes. The supernatant fluid was aspirated away and cell fusion was conducted at 37° C. using polyethylene glycol (PEG) 1500, molecular weight 500-600. The procedure was carried out with constant gentle stirring by addition of the following, at the times indicated: 1.0 ml of 50% PEG in RPMI 1640 added over one minute, with one

100  $\mu$ l of FITC-labelled OKT4 (0.3  $\mu$ g/ml) was incubated in each well overnight and in the dark at 4° C. Wells were washed 10 times with PBS/Tween 20 solution as above. To each well was added 200  $\mu$ l of 50

Formal cloning of hybridoma cells obtained from the positive wells was conducted by diluting the cells into additional wells at a ratio such that there was approximately 1 cell per 3 wells. Generally, formal cloning from an active well produced formal clones which appeared to be subclones of the same hybridoma cell but in several instances revealed different clone populations over three generations. Subclones from the same original well were named with the parent number and an additional number (e.g., clones obtained from well JT4C7 were labeled JT4C7-1, JT4C7-2, etc.).

### A. Relative Affinity Titrations

Correspondingly, Table 2 reveals that of the subclones of JT4C7, clones JT4C7-12 and JT4C7-9 are respectively of the highest and lowest relative affinity in this test procedure.

Concentration Anti-Mouse Antibody ( $\mu\text{g/ml}$ )	Clone No.															
	0 (Blank)	1	2	3	4	5	6	7	8	9	10	11	12	13	16	
10	-27	2882	2799	2511	2913	2529	2435	2601	2395	2523	2463	1003	2281	1151	2203	
3	271	877	803	823	938	723	688	763	617	751	716	405	830	497	620	
1	37	201	200	236	128	178	133	199	84	98	112	92	215	192	272	
0.3	24	31	17	106	37	99	109	142	38	88	76	101	397	185	263	
0.1	205	14	-1	123	23	49	53	103	19	49	61	16	117	70	157	
0.03	392	21	-82	76	68	66	55	120	43	43	125	76	104	107	202	
0.01	301	15	23	105	54	88	46	85	-51	40	103	31	80	60	198	
0.003	17	-8	-43	86	-14	116	42	115	-27	29	126	26	51	65	136	

Subclone No.	Concentration Anti-Mouse Antibody ( $\mu\text{g/ml}$ )										
	3.0	1.5	0.75	0.37	0.18	0.09	0.045	0.022	0.011	0.005	0.002
JT4C7-3	2364	1004	399	337	267	259	217	206	246	215	161



TABLE 2-continued

Subclone No.	Concentration Anti-Mouse Antibody ( $\mu\text{g/ml}$ )										
	3.0	1.5	0.75	0.37	0.18	0.09	0.045	0.022	0.011	0.005	0.002
JT4C7-12	2480	914	376	298	278	262	232	200	249	230	202
JT4C7-8	2420	869	351	291	236	214	235	201	217	188	208
JT4C7-9	2238	924	366	270	289	214	250	211	214	245	251
JT4C7-6	2441	864	344	250	234	210	243	210	203	239	235
JT4C7-4	2372	869	369	275	249	243	233	204	228	227	229
JT4C7-11	2330	828	378	290	268	246	224	211	245	228	227
HAT Medium	786	475	248	306	241	237	245	200	220	242	272

### B. Western Blot Analysis

Antibodies derived from all nineteen positive clones identified in Example 1 were assayed by Western blot analysis for immunoreactivity with proteins of the HIV virion. More specifically, HTLV-III<sub>B</sub> particles were disrupted with SDS (0.1%) and dithiothreitol (0.003M) and the material was placed on a 7.5% polyacrylamide gel, the gel was electrophoresed using standard procedures and materials were transferred to nitrocellulose filter paper. Filters were initially incubated with 20% heat inactivated horse serum in PBS for 1 hour and then washed with PBS. Filters were then incubated with antibody supernatants of each clone for 3 hours at room temperature with gentle shaking and then overnight at 4° C. with gentle shaking. After washing with PBS/Tween 20, and re-blocking with 20% horse serum as above for one hour, 10  $\mu\text{g}$  of peroxidase labelled rabbit anti-mouse IgG was added and the mixture was incubated at room temperature for 4 hours. After 10 washings with PBS/Tween 20, color was developed by standard means. Antibodies derived from clones JT4C8, JT4C12 and JT4C16 all strongly reacted with HIV protein having a molecular weight of about 60,000 to 80,000. The ability of these antibodies to immunoreact with HIV protein that played no part in their generation is strongly predictive of the capacity of these antibodies to effect neutralization of HIV *in vitro* and *in vivo*.

Isotype analysis of the above-noted antibodies reactive in Western blot procedures revealed that JT4C12 and JT4C16 antibodies were of the IgG<sub>3</sub> isotype.

### C. HIV Neutralization

Hybridoma culture supernatants were tested for HIV neutralization capacity in the following manner. Three day growth supernatants were diluted 1:5 in complete medium [500 ml RPMI 1640; 6 ml 100 $\times$ Penicillin/Streptomycin; 6 ml 100 $\times$ L-glutamine; 100 ml FCS; and, 1.2 ml Polybrene Stock (1 mg/ml)]. 200  $\mu\text{l}$  of the medium-diluted sample was added to all but two wells of a 24 well microtiter plate and two wells received an equal quantity of medium alone. Additional medium was added to one of the "medium-only" wells and each remaining well received 200  $\mu\text{l}$  of high titer HIV virus stock. Plates were sealed in plastic bags, incubated for 1-1½ hours at 4° C. and allowed to return to 17° C. upon standing for about 15 minutes. H9 cells were incubated in complete medium for 30 minutes at 37° C. at a density of  $1 \times 10^6$  cells/ml, then centrifuged and resuspended in fresh complete medium at a density of  $5 \times 10^6$  cell/ml. 200  $\mu\text{l}$  of the cell suspension was added to each well (bringing the total volume to 600  $\mu\text{l}$ ) and the plates were incubated for 1 hour at 37° C., whereupon 150  $\mu\text{l}$  was transferred to a duplicate plate containing 2.0 ml of fresh complete medium per well. Cultures were incubated at 37° C. in a CO<sub>2</sub> incubator. After 4 days, the cultures were split and fresh complete medium was

added. At day 7 of incubation samples were prepared for neutralization screening by IFA and Reverse Transcriptase procedures. [See, Guroff et al., *Nature*, 316, 72-74 (1985); Matthews et al., *Proc. Nat'l Acad. Sci. (USA)*, 83, 9709-9713 (1986); and Poiesz et al., *Proc. Nat'l Acad. Sci. (USA)*, 77, 7415-7419 (1980)]. In a first neutralization screening procedure, the results were essentially negative or inconclusive but in a second procedure commenced concurrently with the running of the first, antibodies from 12 of 15 tested clones in the JT4C1-19 series displayed neutralizing activity in the IFA or RT test or both and 5 of 6 antibodies of the JT4C7 subclones tested displayed neutralization characteristics. All neutralization was "partial" in comparison to human AIDS (HTLV-III<sub>B</sub>) patient serum which displayed 100 percent neutralization in these assays.

### EXAMPLE 3

The general hybridoma forming and screening procedures of Example 1 were repeated using the commercially available monoclonal anti-human lymphocyte T4 antibody designated OKT4A (Ortho Diagnostics, Rahway, N.J.) which was purified using a Mono S (rather than Mono Q) column. The specific immunization procedure varied slightly from that of Example 1 in that the initial injection was intraperitoneal and involved approximately 15  $\mu\text{g}$  of purified antibody. The second intraperitoneal inoculation was seven days later and consisted of approximately 10  $\mu\text{g}$  of purified antibody. Thirteen days later, the final booster of about 5  $\mu\text{g}$  of antibody was administered by splenic injection. Of 2090 wells screened, 34 were significantly positive for reaction with OKT4A, with 10 of these displaying high activity (fluorescence values of about 1000 or more against a "background" of approximately 200). These ten positive clones were designated JT1-1D11, JT1-1F3, JT1-1G2, JT1-6E12, JT1-6F12, JT2-8E9, JT3-2C4, JT3-5A11, JT3-6D9, AND JT3-6E8.

### EXAMPLE 4

In order to characterize the antibodies produced by the positive clones described in Example 3, tests were conducted essentially as described in Example 2. Briefly, supernatant antibodies were reactive on the Western blot assay with HIV protein having a molecular weight of from about 60,000 to about 80,000. The assays predominantly indicated reaction with an approximately 67,000 molecular weight protein, with some antibodies showing reactivity with a 78,000 molecular weight species. Significantly no reactivity was noted with 41 Kd or 120 Kd fractions usually characterized as the major immunologically significant HIV envelope glycoproteins. Of the antibodies displaying the strongest reaction, two (JT1-6E12 and JT2-8E9) were of an IgM isotype and the antibody of clone JT1-1F3

was of the IgG<sub>1</sub> isotype. Set out below in Table 3 are the results of the fluorescent-linked assay for employing the JT1-1F3 antibody.

TABLE 3

Concentration Anti-Mouse Antibody (μg/ml)	Blank	Control (HAT)	JT1-1F3
20 μg/ml	4	754	1093
10	-37	738	1010
5	-43	756	899
2.5	-56	759	908
1.25	-2	454	634
0.625	-38	291	325
0.313	23	315	310
0.157	11	340	331

Neutralization studies carried out concurrently with those of Example 2(C) also indicated initial negative neutralization results and, in the second trial, only slight evidence of neutralization capacity on the RT assay. Nonetheless, the IgG<sub>1</sub>-secreting JT1-1F3 clone was selected for ascites amplification and further antibody ELISA screening for reactivity with HIV protein.

In the first ELISA screen, varying concentrations of HTLV-IIIIB protein (respectively, concentrations of 40, 20, 10, 5, 2.5, 1.25, 0.625, 0.313, 0.156, 0.078, 0.039 and 0.020 μg/ml) were coated onto microtiter plates, blocked (20% horse serum/PBS, 37° C., 2 hours), and

African HIV variant designated 906 (obtained from Dr. Jerome Groopman, New England Deaconess Hospital, Boston, Mass.). As graphically illustrated in FIG. 3, the JT1-1F3 antibody recognized a common epitope of all three HIV variants.

Ascites fluid derived JT1-1F3 antibody was then tested for *in vitro* neutralization activity as indicated by the RT assay described above and by syncytium induction. In this procedure a comparison was made between varying levels of JT1-1F3 antibody (ascites fluid IgG fraction), a negative control (ascites fluid/pristane) and a positive control in the form of serum from an HTLV-IIIIB-infected patient. According to this procedure, HIV strains IIIB, AL1212C and 906 were incubated with Mab JR1-1F3, control ascites or neutralizing human serum (in a 1:5 dilution in phosphate buffered saline) for 90 minutes at 4° C. H9 cells (5×10<sup>6</sup>) were then added to each well and incubated for 1 hour at 37° C. Aliquots (150 μl) were removed from each well and added to 2.0 ml fresh medium. The cultures were split 1:1 on day 4. Reverse transcriptase activity and syncytium induction were monitored on day 4 and day 7. The day 7 results of this procedure are provided in Table 4, wherein the relative syncytium induction is indicated as follows: (-), 0/200 cells; (+/-), 1-5/200 cells; (+), >10% cells; (++) , >25% of cells; and (+++), >50% of cells.

TABLE 4

	JT1-1F3 NEUTRALIZATION					
	HIV = HTLV-III B		HIV = AL1212C		HIV = 906	
	RT Activity/ % Neutralization	Syncytium Induction	RT Activity/ % Neutralization	Syncytium Induction	RT Activity/ % Neutralization	Syncytium Induction
1. Non-infected H9	5100/-	-	3420/-	-	4800/-	-
2. HIV	135150/0	+++	317280/0	+++	627510/0	+++
3. HIV + 1 mg/ml Ab	5730/100	-	12030/96.2	-	56970/90.9	-
4. HIV + 600 μg/ml Ab	50700/62.4	-	130380/58.9	+/-	431670/31.2	-
5. HIV + 300 μg/ml Ab	78330/42.0	++	240030/24.3	+	497850/20.6	+/-
6. HIV + 50 μg/ml Ab	132780/1.7	+++	198690/37.4	+++	647670/0	++
7. HIV + Ascites (control)	168000/0	+++	281100/11.4	+++	765000/0	+++
8. HIV + Human Serum	5460/100	-	197130/37.8	++	688680/0	+++

dried overnight. Culture supernatant (20 μl with 80 μl PBS) or HAT medium control was added as a first antibody. After one hour of incubation at room temperature and storage at 4° C. overnight, peroxidase conjugated rabbit anti-mouse IgG (0.3 μg/ml in 5% horse serum/PBS) was added as the second antibody. After 2 hours of incubation at room temperature, substrate was added (O-phenylenediamine, 0.5 mg/ml in McIlvan's Buffer, pH 5.5; 10 ml of 3% hydrogen peroxide). After 20 min. of incubation at room temperature, the reaction was stopped with 50 μl 0.4M sulfuric acid and absorbance was read at 492-610. The results of the test are graphically represented in FIG. 1 and indicate that HIV was detectable at levels of 1.25 μg and also that reactivity progressively increased with up to 40 μg of virus.

The ELISA procedure was repeated on plates initially coated with varying concentrations of uninfected H9 cell membrane preparations [10<sup>6</sup>, 6×10<sup>5</sup>, 4×10<sup>5</sup>, 2×10<sup>5</sup>, 6×10<sup>4</sup>, 4×10<sup>4</sup>, 2×10<sup>4</sup>, 1×10<sup>4</sup>, 6×10<sup>3</sup>, 4×10<sup>3</sup>, 2×10<sup>3</sup> cells/ml]. The results of this test procedure are graphically represented in FIG. 2 and indicate no reactivity with non-infected preparations.

Finally, the HIV protein ELISA was repeated using the same varying concentrations of protein from the HTLV-IIIIB HIV variant, the Haitian HIV variant designated AL1212C (obtained from Dr. David Hall, Massachusetts General Hospital, Boston, Mass.) and the

The results shown in Table 4 clearly demonstrate *in vitro* neutralizing activity for the JT1-1F3 antibody versus all three HIV variants tested, in contrast to the neutralizing activity for infected patient serum, which was completely variant-specific, indicating recognition by the monoclonal antibody of a significant type-common epitope.

The molecular weight of the JT1-1F3 antibody reactive species was determined by immunoblotting. Purified HTLV-IIIIB (5 and 10 μg) was analyzed by SDS-PAGE and Coomassie blue/silver staining (FIG. 4, Lanes B and C). Similar aliquots were analyzed by SDS-PAGE, transferred to nitrocellulose paper and analyzed for reactivity with JT1-1F3 antibody. A single reactive species was detectable at approximately 67 kd (FIG. 4, Lanes E and F).

Reactivity of JT1-1F3 antibody with the HTLV-IIIIB isolate was compared to that with other HIV strains. Similar patterns of reactivity by ELISA were obtained with the HTLV-IIIIB, AL1212C and 906 strains. Furthermore, Western blot analysis with JT1-1F3 antibody and each of the three HIV strains revealed reactivity with antigens of similar molecular weight (FIG. 5). These findings indicate that Mab JT1-1F3 reacts with

related or identical antigens detectable in divergent HIV strains.

The above findings with JT1-1F3 antibody suggested that it might also be useful in detecting HIV virus-infected cells. In this regard, the binding of JT1-1F3 antibody (ascites fluid IgG fraction) to uninfected and HIV-infected H9 cells was monitored. The extent of JT1-1F3 binding was determined by a fluoresceinated rabbit anti-mouse IgG. As shown in FIG. 6A, there was little if any detectable binding of antibody to uninfected H9 cells. In contrast, focal and diffuse binding of the antibody was detectable with HTLV-III-B-infected H9 cells (FIG. 6B). Similar findings were obtained when using H9 cells infected with the 906 and AL1212C strains (FIGS. 6C and D). This approach has been extended to hematopoietic cells from a patient with AIDS. Mononuclear cells were collected from peripheral blood of the AIDS patient by Ficoll-Hypaque separation and examined for reactivity with JT1-1F3. As shown in FIG. 6E, a focal and diffuse immunofluorescent staining pattern was detected with these cells that was similar to the findings obtained with HIV-infected H9 cells.

It is noteworthy that screening of the JT1-1F3 antibody for reactivity with cells possessing class II major histocompatibility ("MHC") surface components (i.e., human B cell line MD1 and normal peripheral blood mononuclear cells) reveal no substantial reactivity.

Subcloning of the JT1-1F3 resulted in the selection of subclones JT1-1F3-E5 and JT1-1D7 as respectively producing progressively higher levels of IgG1 antibody than JT1-1F3. Comparative neutralization assay data for JT1-1F3, JT1-1F3-E5 and JT1-1D7 is set out in Table 5 below. Reactivity within the ELISA assay format for the antibodies produced by these three hybridomas is set out in Table 6 below.

TABLE 5

NEUTRALIZATION			
	HIV = HTLV-III B RT Activity/ % Neutralization	HIV = AL1212C RT Activity/ % Neutralization	HIV = 906 RT Activity/ % Neutralization
<b>JT1-1F3</b>			
1. Non-infected H9	464/—	455/0	494/0
2. HIV	10976/0	58131/0	127317/0
3. HIV + 4 mg/ml Ab	5823/49.0	15754/73.5	27212/78.9
4. HIV + 2 mg/ml Ab	8561/23.0	47571/18.3	156653/0
5. HIV + 400 µg/ml Ab	7183/36.1	515261/11.5	1803291/0
<b>JT1-1F3-E5</b>			
1. Non-infected H9	126/0	121/—	126/—
2. HIV	10990/0	33501/0	16213/0
3. HIV + 2.9 mg/ml Ab	NT*	9281/72.6	NT
4. HIV + 1.9 mg/ml Ab	NT	11559/65.7	—
5. HIV + 1 mg/ml Ab	552/96.1	17967/46.5	2253/86.8
6. HIV + 500 µg/ml Ab	775/94.0	NT	3894/76.6
<b>JT1-1D7</b>			
1. Non-infected H9	214/0		
2. HIV	10998/0		
3. HIV + 500 µg/ml Ab	177/100		
4. HIV + 250 µg/ml Ab	1549/87.6		

\*NT = Not Tested

TABLE 6

Comparison of 3 Hybridoma on ELISA				
HIV-III B Viral Protein	JT1-1F3	JT1-1E5	JT1-1D7	Control (Culture Media)
10	1.337	1.111	1.392	0.031
5	0.914	0.887	1.118	0.023
2.5	0.996	0.831	1.081	0.022
1.25	0.499	0.278	0.523	0.011
0.63	0.221	0.122	0.176	0.020

TABLE 6-continued

Comparison of 3 Hybridoma on ELISA				
HIV-III B Viral Protein	JT1-1F3	JT1-1E5	JT1-1D7	Control (Culture Media)
0.31	0.070	0.075	0.060	0.015
0.16	0.048	0.049	0.036	0.004
0.08	0.027	0.027	0.031	0.012

## EXAMPLE 5

The hybridoma forming and screening procedures of Examples 1 and 3 were again repeated using OKT4A, with the following variations in the immunization procedure. The initial immunization involved intraperitoneal injection of 10 µg of Mono S purified OKT4A antibody; the second intraperitoneal injection (7 µg) was given seventeen days later; and the final, 7 µg intravenous dose was administered 18 days later. After fusion and screening, an IgG<sub>1</sub>-producing positive clone, designated JT2-N15 was selected for further study. Like JT1-1F3 and its subclones JT1-1F3-E5 and JT1-1D7, clone JT2-N15 produced a monoclonal antibody which was reactive on the Western blot assay with HIV protein having a molecular weight of about 65–67,000. Culture media from growth of JT2-N15 was positive in the ELISA assay and the results in a preliminary neutralization assay with respect to HIV-III B infectivity are set out in Table 7 below.

TABLE 7

Neutralization of HIV-III B By JT2-N15	
	RT Activity and % Neutralization
1. Non-infected H9	157/—
2. HIV	12194/0
3. HIV + 1.5 mg/ml Ab	343/98.5

60 4. HIV + 750 µg/ml Ab 568/96.6

In further screening procedures for neutralizing activity of anti-idiotypic antibodies propagated by the ascites method, it has been preliminarily determined that the following protocol generates the most active ascites preparation. Five to eight week old mice are "primed" with 0.5 to 1.0 ml Pristane and two weeks later injected with 2 to 8 × 10<sup>6</sup> (preferably about 5 × 10<sup>6</sup>)

hybridoma cells. Collection of ascites fluids commenced two weeks after inoculation and the initial fluids collected (about 3-5 ml) displayed the highest neutralization activity. A second collection of ascites fluid carried out three days later produced from 8 to 10 ml of fluid having lesser activity. A third collection from surviving animals generally provided 3-5 ml of fluid which, at times, was substantially less active than either of the materials from the first and second collection. Neutralization data shown for JT1-1F3 in Tables 4 and 5 was based on ascites pooled from three collections whereas data for JT1-1F3-E5, JT1-1D7 and JT2-N15 in Tables 5 and 7 was based on ascites materials pooled from first and second collections only.

While the foregoing illustrative examples have been directed to procedures involving the commercially available monoclonal antibody preparations OKT4 and OKT4A, other commercial antibodies such as Leu3a (Becton-Dickenson, Immunocytometry Systems, Mountain View, Calif., 94039) are expected to be equally suitable for use in generating anti-idiotypic antibodies according to the invention. Equally suitable are non-commercial antibodies (preferably monoclonal antibodies) prepared by known hybridoma techniques using human T cells, human T lymphoblast cells which express the T4 glycoprotein and recombinant-produced human T4 protein isolates as the initial immunogen. Moreover, while antibodies produced by JT1-1F3 and its subclones and JT2-N15 are of the IgG<sub>1</sub> isotype, it is expected that antibodies of differing isotypes will be equally useful. Antibodies of the IgG<sub>2</sub> isotype, for example, may be more useful in procedures involving complement mediated cytolytic reactions.

Confirmation of the operability of the procedures of the present invention is provided by the reports of Chanh et al., *P.N.A.S.(USA)*, 84, 3891-3895 (June, 1987) wherein it is reported that monoclonal antibody to Leu3a (designated HF1.7) was capable of *in vitro* neutralization of HIV-IIIB infectivity. However, neutralizing activity of HF1.7 has been characterized as "weak" [see Weiss, *Nature*, 331, p. 15 (January, 1988)] and has not been demonstrated to extend to strains other than HIV-IIIB. Moreover, unlike the anti-OKT4 and OKT4A monoclonal antibodies of the foregoing examples, the anti-Leu3a antibody of Chanh et al. is not indicated as reactive with any HIV-derived protein other than gp120 as shown in FIG. 4 at page 3894 of Chanh et al., *supra*. [See also, Dagleish et al., *The Lancet*, ii, 1047-1050, Nov. 7, 1987, relating to polyclonal anti-Leu3a antibodies.]

While the foregoing examples relate to murine-derived hybridoma cell preparations, it is within the contemplation of the invention to generate and employ hybrid hybridomas (e.g., mouse/human) and especially human/human hybridomas prepared, for example, in a manner consistent with Borrebaeck, *TIBTECH*, June, 1986, p. 147-153; Abrams et al., *Methods in Enzymology*, 121, pp. 107-119 (1986); Kozbor et al., *Methods in Enzymology*, 121, pp. 120-140 (1986); Suresh et al., *Methods in Enzymology*, 121, pp. 210-228 (1986); and Masuho et al., *Biochem. & Biophys. Res. Comm.*, 135(2), pp. 495-500 (1986). See, also, Klausner, "Single Chain Antibodies Become a Reality", *Bio/Technology*, 4, 1042-1045 (1986); Klausner, "Stage Set For 'Immunological Star Wars'", *Bio/Technology*, 5, 867-868 (1987) and Marx, "Antibodies Made To Order", *Science*, 229, 455-456 (1985).

It will be readily understood, therefore, that the above specific illustrative methodologies for the production of hybridomas and the identification and isolation of monoclonal antibodies are not intended to be restrictive of the scope of practice of the invention. Numerous alternative methodologies exist for achieving the same results as demonstrated, for example, by articles appearing in *Methods in Enzymology*, Vol. 121, "Immunochemical Techniques, Part I", Langone et al., eds., Academic Press, Inc. (New York, 1986).

It is also within the contemplation of the invention to develop immunologically active polypeptides for use in diagnostic and therapeutic methods of the invention by means of expression of DNA sequences encoding therefor in suitably transformed or transfected procaryotic and eucaryotic host cells in culture.

Anti-HIV therapeutic methods of the invention will be understood to comprise the administration of effective amounts of antibodies or antibody fragments of the invention to a patient infected with HIV or at risk of HIV infection in order to generate passive immunity involving neutralizing infectivity of HIV *in vivo*. In this regard, combination of products of the invention with immunologically acceptable diluents, adjuvants and carriers is contemplated in order to form immunologically effective anti-HIV therapeutic compositions.

Anti-HIV therapeutic methods of the invention within the contemplation of the invention also comprehend active immunization using biologically active HIV protein fractions reactive with the monoclonal anti-monoclonal anti-human lymphocytes. Such products may be obtained directly from virus preparations by well known affinity purification processes involving forming immunological reaction mixtures between HIV proteins and antibodies of the invention, followed by isolation of the desired protein. As one example, the 60,000 to 80,000 molecular weight HIV protein recognized by the JT1-1F3 in procedures of Example 4 is a prime candidate for vaccine use. The same is expected to be true for recombinant expression products based on HIV DNA which can be immunologically identified (and/or purified) by means of antibodies of the invention.

Diagnostic methods of the invention wherein polypeptide products are employed to detect and quantify HIV particles in biological fluids such as blood are expected to form an essential part in preliminary screening for patients who would benefit from passive immunization according to the invention and in the monitoring therapeutic regimens of the invention.

The finding that antibodies of the invention are selectively reactive with the surfaces of HIV-infected cells is indicative of a variety of diagnostic and therapeutic utilities including histological screening of tissue (e.g., lymphatic cells) and fluid (e.g., blood) samples for detection of HIV infection, possibly at early stages of infection not readily detectable by assays based on screening for antibodies. Recognition of infected cells by antibodies of the invention allows for segregation of such cells from cell populations comprising both infected and non-infected cells. It is thus contemplated that blood of AIDS patients may be subjected to extracorporeal treatment to remove or selectively kill infected lymphocytes through use of antibodies of the invention. Moreover, within the contemplation of the invention is *in vivo* treatment with antibodies of the invention coupled with supplementation of circulating complement to effect cytolysis of infected cells. Sup-

port for the operability of such a therapeutic protocol is provided by preliminary positive results of tests for the capacity of JT1-1D7 antibodies to participate in the *in vitro* complement dependent cytolysis of HIV infected H9 cells.

Selective reactivity properties of antibodies make them good candidates for *in vivo* drug or toxin delivery to infected cells and use in development of bi-specific antibodies which will include double determinants allowing for, e.g., "focusing" effector T cell activity. See, e.g., Staerz et al., *Proc. Nat'l Acad. Sci. (USA)*, 83, 1453-1457 (1986).

Based on the fact that the present invention has its foundation in the immunological characteristics of T4 protein—which is believed to provide the common receptor for HIV infection by all variants (e.g., AL1212C, 906, ARC, LAV, HTLV-III<sub>RF</sub>, HTLV-III<sub>B</sub>)—rather than any specific HIV variant, it is expected that diagnostic and therapeutic methods of the invention will be applicable to detection and treatment of infection involving all HIV variants. Use of polypeptides of the invention as diagnostic and research tools is expected to provide additional information into the nature of the interaction between HIV and host cells. As one example, monoclonal antibodies of the invention will be useful in identifying the precise primary, secondary and tertiary structural conformation of region(s) of surface proteins of HIV and host cells which are specifically and necessarily interactive in the recognition and association processes involved in HIV infection of cells. This information, in turn, would allow for generation of immunologically active materials of the invention through use of synthetic and recombinant-produced peptide immunogens.

The foregoing detailed description has been given for clearness of understanding only and no unnecessary limitations should be understood therefrom inasmuch as numerous modifications and variations will be expected to occur to those skilled in the art.

What is claimed is:

1. In an assay procedure for detection and/or quantification of HIV in a biological fluid based upon an immunological reaction between HIV and an immunologically reactive polypeptide capable of specific immunobinding to HIV, the improvement comprising reacting said biological fluid with a purified and isolated immunologically active polypeptide in the form of an

antibody or chimeric antibody or antibody fragment capable of specific immunobinding with that portion of the HIV virion which is necessarily interactive with T4 surface proteins during HIV infection of host cells and characterized by the capacity to neutralize infectivity of HIV *in vitro* and by specific immunoreactivity with an antibody immunospecific for human lymphocyte T4.

2. In an assay procedure for detection and/or quantification of HIV in a biological fluid based upon an immunological reaction between HIV and an antibody capable of specific immunobinding to HIV, the improvement comprising reacting said biological fluid with a monoclonal anti-monoclonal-anti-human lymphocyte T4 antibody.

3. An assay procedure for detection and/or quantification of HIV-infected host cells in a fluid or tissue sample, said method comprising: forming an immunological reaction mixture of said sample with a purified and isolated immunologically active polypeptide in the form of an antibody or chimeric antibody or antibody fragment capable of specific immunobinding with that portion of the HIV virion which is necessarily interactive with T4 surface proteins during HIV infection of host cells and characterized by the capacity to neutralize infectivity of HIV *in vitro* by specific immunoreactivity with an antibody immunospecific for human lymphocyte T4 and detecting the presence of immunobinding of said polypeptide to surfaces of infected cells.

4. An assay procedure for detection and/or quantification of HIV-infected host cells in a fluid or tissue sample, said method comprising: forming an immunological reaction mixture of said sample with a monoclonal antimonoclonal-anti-human lymphocyte T4 antibody and detecting the presence of immunobinding of said antibody to surfaces of infected cells.

5. An assay according to claim 3 wherein said detection step comprises determination of a detectable label bound to said antibody.

6. An assay according to claim 3 wherein said detection step comprises determination of a detectable label bound to an antibody raised against said antibody added to said reaction mixture.

7. An assay according to claim 3 wherein said antibody is selected from the group consisting of anti-OKT4 antibody and anti-OKT4A antibody.

\* \* \* \* \*

UNITED STATES PATENT AND TRADEMARK OFFICE  
CERTIFICATE OF CORRECTION

PATENT NO. : 5,169,752

Page 1 of 2

DATED : December 8, 1992

INVENTOR(S) : Tsuneya Ohno

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

At Column 3, line 1, after protein insert ---.

At Column 5, line 19, replace "graphiclly" with --graphically--.

At Column 5, line 46, replace "neutralizaitn" with --neutralization--.

At Column 6, line 16, replace "miligram" with --milligram--.

At Column 7, line 35, replace "IgGFc" with --IgG Fc--.

At Column 9, line 49, replace "Streptomyucins;" with --Streptomycins;--.

At Column 12, line 1, replace "form" with --from--.

At Column 13, line 31, replace "IgG1" with --IgG<sub>1</sub>--.

At Column 15, line 34, replace "complement mediated" with  
--complement-mediated--.

At Column 15, line 52, replace "murinede-rived" with --murine-derived--.

At Column 18, line 37, after "3" insert --or 4--.

At Column 18, line 40, after "3" insert --or 4--.

UNITED STATES PATENT AND TRADEMARK OFFICE  
**CERTIFICATE OF CORRECTION**

PATENT NO. : 5,169,752

Page 2 of 2

DATED : December 8, 1992

INVENTOR(S) : Tsuneya Ohno

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 18, line 44, after "3" insert --or 4--

Signed and Sealed this  
Twelfth Day of April, 1994



Attest:

BRUCE LEHMAN

Commissioner of Patents and Trademarks

Attesting Officer



**(10) Patent No.: US 6,818,740 B1**  
**(45) Date of Patent: Nov. 16, 2004**

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(List continued on next page.)

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## ABSTRACT

**Inhibitors of HIV membrane fusion and a method of identifying drugs or agents which inhibit binding of the N-helix coiled-coil and the C helix of HIV gp41 envelope protein.**

**2 Claims, 45 Drawing Sheets**

### Discussion Summary

[illegible]

G = glycine  
 A = alanine  
 C = cysteine  
 D = aspartic acid  
 E = glutamic acid  
 U = glutamine  
 W = tryptophan  
 F = phenylalanine  
 H = histidine  
 I = isoleucine  
 Q = glutamine



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Figure 1: HIV-1 gp41 Structure and Peptides

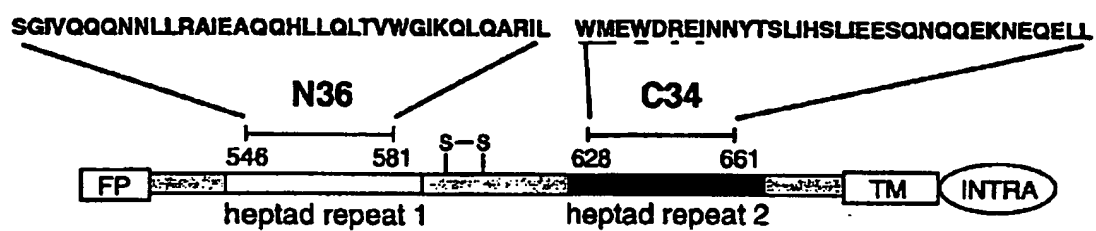


Figure 2: Correlation of C34 Inhibitory Potency With N36/C34 Stability

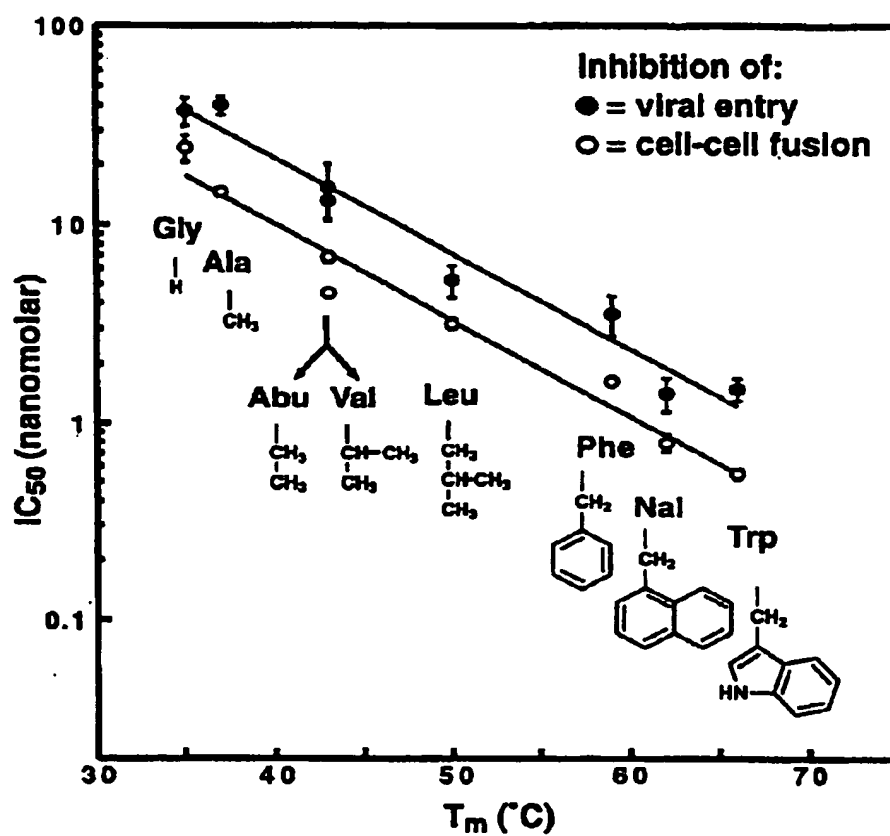


Figure 3: D-peptide Sequences

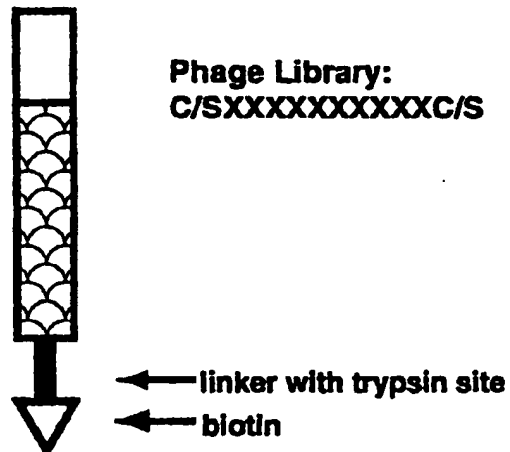
D10pep1 :       Ac- G A C E A R H R E W A W L C A A - CONH2  
D10pep1a:   Ac - KK G A C E A R H R E W A W L C A A - CONH2  
  
D10pep3 :   Ac - KK G A C G L G Q E E W F W L C A A - CONH2  
  
D10pep4 :       Ac - G A C D L K A K E W F W L C A A - CONH2  
  
D10pep5 :   Ac - KK G A C E L L G W E W A W L C A A - CONH2  
D10pep5a: Ac - KKKK G A C E L L G W E W A W L C A A - CONH2  
  
D10pep6 :       Ac - G A C S R S Q P E W E W L C A A - CONH2  
D10pep6a :   Ac - KK G A C S R S Q P E W E W L C A A - CONH2  
  
D10pep7a:   Ac - KK G A C L L R A P E W G W L C A A - CONH2  
  
D10pep10:   Ac - KK G A C M R G E W E W S W L C A A - CONH2  
  
D10pep12:   Ac - K K G A C P P L N K E W A W L C A A - CONH2  
  
Consensus Sequence       C X X X X X E W X W L C

## Where:

G = glycine  
A = alanine  
C = cysteine  
D = aspartic acid  
L = leucine  
K = lysine  
E = glutamic acid  
W = tryptophan  
F = phenylalanine  
R = arginine  
H = histidine  
S = serine  
Q = glutamine

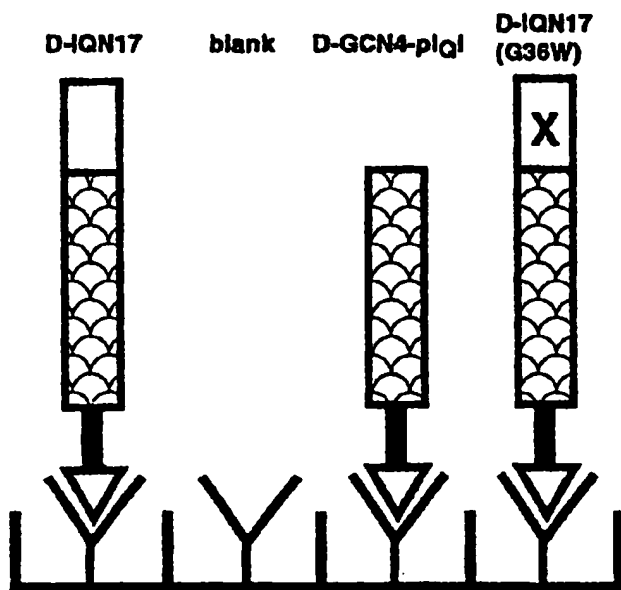
**Figure 4: Mirror-Image Phage Display with the D-IQN17 Target**

1. Perform rounds of phage selection to identify binders to D-IQN17.

**D-IQN17**

2. Sequence individual phage clones

3. Test for specificity of binding. Determine if the phage bind to the gp41 region of D-IQN17.



4. Synthesize D-peptides.
5. Assay anti-HIV activity of D-peptides.

## Relationship of D-peptides to IQN17

Figure 5A

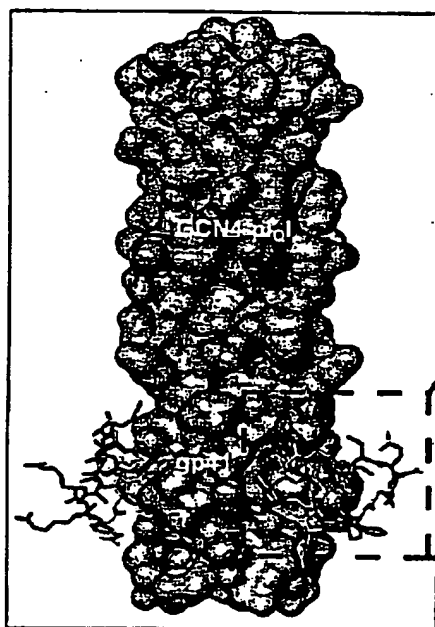


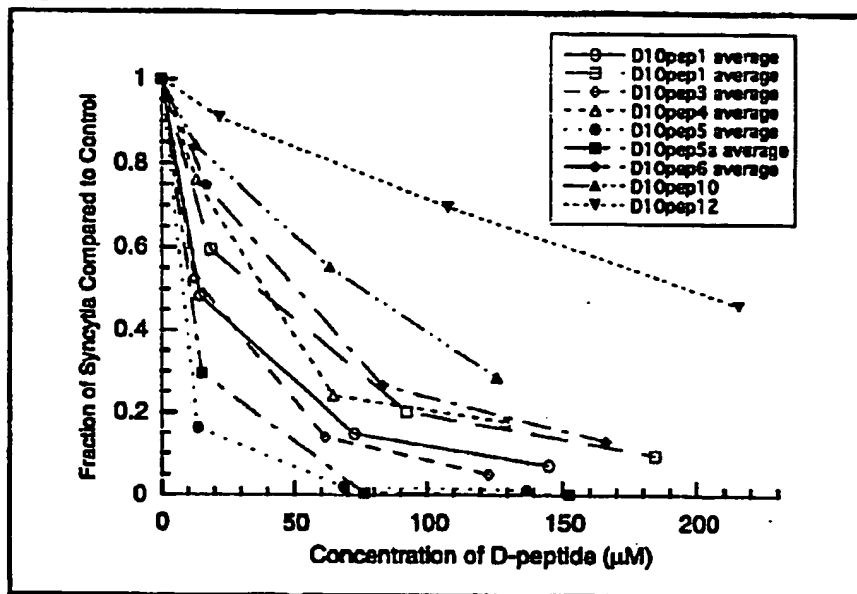
Figure 5B





## Syncytia Assays

Figure 6A

Figure 6B: IC<sub>50</sub> Data for D-Peptides:

D-Peptide	Approximate IC <sub>50</sub> Value (from one or more experiments)
D10pep1	$2 \times 10^{-5}$ M
D10pep1A	$3 \times 10^{-5}$ M
D10pep3	$1 \times 10^{-5}$ M
D10pep4	$3 \times 10^{-5}$ M
D10pep5	$3 \times 10^{-6}$ M
D10pep5a	$6 \times 10^{-6}$ M
D10pep6	$3 \times 10^{-5}$ M
D10pep7a	$4 \times 10^{-5}$ M
Dpep10	$6 \times 10^{-5}$ M
Dpep12	$2 \times 10^{-4}$ M

D10pep3 } show anti-viral effects  
 D10pep4 } with IC<sub>50</sub> values of  
 D10pep5 } less than  $1 \times 10^{-4}$  M.

REMARK 3  
REMARK 3 REFINEMENT.  
REMARK 3 PROGRAM : CNS 0.5  
REMARK 3 AUTHORS : BRUNGER, ADAMS, CLORE, DELANO,  
REMARK 3 GROS, GROSSE-KUNSTLEVE, JIANG,  
REMARK 3 KUSZEWSKI, NILGES, PANNU, READ,  
REMARK 3 RICE, SIMONSON, WARREN  
REMARK 3  
REMARK 3 DATA USED IN REFINEMENT.  
REMARK 3 RESOLUTION RANGE HIGH (ANGSTROMS) : 1.50  
REMARK 3 RESOLUTION RANGE LOW (ANGSTROMS) : 10.00  
REMARK 3 DATA CUTOFF (SIGMA(F)) : 0.0  
REMARK 3 DATA CUTOFF HIGH (ABS(F)) : 646169.44  
REMARK 3 DATA CUTOFF LOW (ABS(F)) : 0.000000  
REMARK 3 COMPLETENESS (WORKING+TEST) (%) : 94.6  
REMARK 3 NUMBER OF REFLECTIONS : 13549  
REMARK 3  
REMARK 3 FIT TO DATA USED IN REFINEMENT.  
REMARK 3 CROSS-VALIDATION METHOD : THROUGHOUT  
REMARK 3 FREE R VALUE TEST SET SELECTION : RANDOM  
REMARK 3 R VALUE (WORKING SET) : 0.214  
REMARK 3 FREE R VALUE : 0.245  
REMARK 3 FREE R VALUE TEST SET SIZE (%) : 10.1  
REMARK 3 FREE R VALUE TEST SET COUNT : 1362  
REMARK 3 ESTIMATED ERROR OF FREE R VALUE : 0.007  
REMARK 3  
REMARK 3 FIT IN THE HIGHEST RESOLUTION BIN.  
REMARK 3 TOTAL NUMBER OF BINS USED : 6  
REMARK 3 BIN RESOLUTION RANGE HIGH (A) : 1.50  
REMARK 3 BIN RESOLUTION RANGE LOW (A) : 1.59  
REMARK 3 BIN COMPLETENESS (WORKING+TEST) (%) : 96.1  
REMARK 3 REFLECTIONS IN BIN (WORKING SET) : 2008  
REMARK 3 BIN R VALUE (WORKING SET) : 0.233  
REMARK 3 BIN FREE R VALUE : 0.270  
REMARK 3 BIN FREE R VALUE TEST SET SIZE (%) : 9.8  
REMARK 3 BIN FREE R VALUE TEST SET COUNT : 219  
REMARK 3 ESTIMATED ERROR OF BIN FREE R VALUE : 0.018  
REMARK 3  
REMARK 3 NUMBER OF NON-HYDROGEN ATOMS USED IN REFINEMENT.  
REMARK 3 PROTEIN ATOMS : 0  
REMARK 3 NUCLEIC ACID ATOMS : 0  
REMARK 3 HETEROGEN ATOMS : 0  
REMARK 3 SOLVENT ATOMS : 0  
REMARK 3  
REMARK 3 B VALUES.  
REMARK 3 FROM WILSON PLOT (A\*\*2) : 21.6  
REMARK 3 MEAN B VALUE (OVERALL, A\*\*2) : 29.7  
REMARK 3 OVERALL ANISOTROPIC B VALUE.  
REMARK 3 B11 (A\*\*2) : 3.61  
REMARK 3 B22 (A\*\*2) : 3.61  
REMARK 3 B33 (A\*\*2) : -7.22  
REMARK 3 B12 (A\*\*2) : 1.74  
REMARK 3 B13 (A\*\*2) : 0.00  
REMARK 3 B23 (A\*\*2) : 0.00  
REMARK 3  
REMARK 3 BULK SOLVENT MODELING.  
REMARK 3 METHOD USED : FLAT MODEL  
REMARK 3 KSOL : 0.394054

Figure 7A

```
REMARK 3 BSOL : 58.3445 (A**2)
REMARK 3
REMARK 3 ESTIMATED COORDINATE ERROR.
REMARK 3 ESD FROM LUZZATI PLOT (A) : 0.18
REMARK 3 ESD FROM SIGMAA (A) : 0.09
REMARK 3 LOW RESOLUTION CUTOFF (A) : 5.00
REMARK 3
REMARK 3 CROSS-VALIDATED ESTIMATED COORDINATE ERROR.
REMARK 3 ESD FROM C-V LUZZATI PLOT (A) : 0.20
REMARK 3 ESD FROM C-V SIGMAA (A) : 0.12
REMARK 3
REMARK 3 RMS DEVIATIONS FROM IDEAL VALUES.
REMARK 3 BOND LENGTHS (A) : 0.012
REMARK 3 BOND ANGLES (DEGREES) : 1.5
REMARK 3 DIHEDRAL ANGLES (DEGREES) : 15.7
REMARK 3 IMPROPER ANGLES (DEGREES) : 1.00
REMARK 3
REMARK 3 ISOTROPIC THERMAL MODEL : RESTRAINED
REMARK 3
REMARK 3 ISOTROPIC THERMAL FACTOR RESTRAINTS. RMS SIGMA
REMARK 3 MAIN-CHAIN BOND (A**2) : 0.956 ; 2.0
REMARK 3 MAIN-CHAIN ANGLE (A**2) : 1.503 ; 3.0
REMARK 3 SIDE-CHAIN BOND (A**2) : 1.853 ; 3.0
REMARK 3 SIDE-CHAIN ANGLE (A**2) : 2.676 ; 3.5
REMARK 3
REMARK 3 NCS MODEL : NONE
REMARK 3
REMARK 3 NCS RESTRAINTS. RMS SIGMA/WEIGHT
REMARK 3 GROUP 1 POSITIONAL (A) : NULL ; NULL
REMARK 3 GROUP 1 B-FACTOR (A**2) : NULL ; NULL
REMARK 3
REMARK 3 PARAMETER FILE 1 : protein_rep_d.param
REMARK 3 PARAMETER FILE 2 : CNS_TOPPAR/water_rep.param
REMARK 3 PARAMETER FILE 3 : CNS_TOPPAR/ion.param
REMARK 3 TOPOLOGY FILE 1 : CNS_TOPPAR/protein.top
REMARK 3 TOPOLOGY FILE 2 : CNS_TOPPAR/water.top
REMARK 3 TOPOLOGY FILE 3 : CNS_TOPPAR/ion.top
REMARK 3
REMARK 3 OTHER REFINEMENT REMARKS: NULL
SEQRES 1 A 214 ACE ARG MET LYS GLN ILE GLU ASP LYS ILE GLU GLU ILE
SEQRES 2 A 214 GLU SER LYS GLN LYS LYS ILE GLU ASN GLU ILE ALA ARG
SEQRES 3 A 214 ILE LYS LYS LEU LEU GLN LEU THR VAL TRP GLY ILE LYS
SEQRES 4 A 214 GLN LEU GLN ALA ARG ILE LEU ACE DLY DLA DCS DLU DLA
SEQRES 5 A 214 DRG DIS DRG DLU DRP DLA DRP DEU DCS DLA DLA CL WAT
SEQRES 6 A 214 WAT WAT WAT WAT WAT WAT WAT WAT WAT WAT WAT WAT WAT
SEQRES 7 A 214 WAT WAT WAT WAT WAT WAT WAT WAT WAT WAT WAT WAT WAT
SEQRES 8 A 214 WAT WAT WAT WAT WAT WAT WAT WAT WAT WAT WAT WAT WAT
SEQRES 9 A 214 WAT WAT WAT WAT WAT WAT WAT WAT WAT WAT WAT WAT WAT
SEQRES 10 A 214 WAT WAT WAT WAT WAT WAT WAT WAT WAT WAT WAT WAT WAT
SEQRES 11 A 214 WAT WAT WAT WAT WAT WAT WAT WAT WAT WAT WAT WAT WAT
SEQRES 12 A 214 WAT WAT WAT WAT WAT WAT WAT WAT WAT WAT WAT WAT WAT
SEQRES 13 A 214 WAT WAT WAT WAT WAT WAT WAT WAT WAT WAT WAT WAT WAT
SEQRES 14 A 214 WAT WAT WAT WAT WAT WAT WAT WAT WAT WAT WAT WAT WAT
SEQRES 15 A 214 WAT WAT WAT WAT WAT WAT WAT WAT WAT WAT WAT WAT WAT
SEQRES 16 A 214 WAT WAT WAT WAT WAT WAT WAT WAT WAT WAT WAT WAT WAT
SEQRES 17 A 214 WAT WAT WAT WAT WAT WAT
CRYST1 41.829 41.829 84.817 90.00 90.00 120.00 P 3 2 1 6
ORIGX1 1.000000 0.000000 0.000000 0.000000
```

Figure 7B

ORIGX2	0.000000	1.000000	0.000000	0.000000					
ORIGX3	0.000000	0.000000	1.000000	0.000000					
SCALE1	0.023907	0.013803	0.000000	0.000000					
SCALE2	0.000000	0.027605	0.000000	0.000000					
SCALE3	0.000000	0.000000	0.011790	0.000000					
ATOM	1	CA	ACE	A	0	26.930	7.813	-22.925	1.00 54.89 A
ATOM	2	C	ACE	A	0	26.773	9.004	-22.017	1.00 54.85 A
ATOM	3	O	ACE	A	0	25.855	9.820	-22.124	1.00 54.90 A
ATOM	4	N	ARG	A	1	27.749	9.121	-21.117	1.00 54.75 A
ATOM	5	CA	ARG	A	1	27.815	10.229	-20.165	1.00 54.58 A
ATOM	6	CB	ARG	A	1	27.625	11.568	-20.887	1.00 54.54 A
ATOM	7	CG	ARG	A	1	27.841	12.790	-20.010	1.00 54.10 A
ATOM	8	CD	ARG	A	1	27.657	14.085	-20.800	1.00 54.18 A
ATOM	9	NE	ARG	A	1	28.177	15.253	-20.086	1.00 54.02 A
ATOM	10	CZ	ARG	A	1	29.470	15.495	-19.870	1.00 54.03 A
ATOM	11	NH1	ARG	A	1	30.395	14.654	-20.312	1.00 53.79 A
ATOM	12	NH2	ARG	A	1	29.843	16.587	-19.206	1.00 53.77 A
ATOM	13	C	ARG	A	1	26.752	10.087	-19.074	1.00 54.54 A
ATOM	14	O	ARG	A	1	27.042	10.224	-17.884	1.00 54.51 A
ATOM	15	N	MET	A	2	25.518	9.809	-19.480	1.00 54.42 A
ATOM	16	CA	MET	A	2	24.445	9.671	-18.515	1.00 54.44 A
ATOM	17	CB	MET	A	2	23.074	9.796	-19.202	1.00 54.68 A
ATOM	18	CG	MET	A	2	22.749	8.736	-20.238	1.00 54.76 A
ATOM	19	SD	MET	A	2	21.345	9.252	-21.275	1.00 55.63 A
ATOM	20	CE	MET	A	2	22.189	9.658	-22.822	1.00 55.29 A
ATOM	21	C	MET	A	2	24.557	8.360	-17.755	1.00 54.51 A
ATOM	22	O	MET	A	2	24.073	8.249	-16.629	1.00 54.42 A
ATOM	23	N	LYS	A	3	25.208	7.372	-18.362	1.00 54.34 A
ATOM	24	CA	LYS	A	3	25.383	6.082	-17.702	1.00 54.29 A
ATOM	25	CB	LYS	A	3	26.212	5.139	-18.581	1.00 54.05 A
ATOM	26	CG	LYS	A	3	26.527	3.786	-17.956	1.00 54.04 A
ATOM	27	CD	LYS	A	3	27.727	3.853	-17.018	1.00 54.12 A
ATOM	28	CE	LYS	A	3	28.108	2.469	-16.513	1.00 54.37 A
ATOM	29	NZ	LYS	A	3	29.332	2.493	-15.656	1.00 53.92 A
ATOM	30	C	LYS	A	3	26.097	6.344	-16.384	1.00 54.33 A
ATOM	31	O	LYS	A	3	25.779	5.740	-15.353	1.00 54.60 A
ATOM	32	N	GLN	A	4	27.064	7.255	-16.426	1.00 53.94 A
ATOM	33	CA	GLN	A	4	27.811	7.626	-15.236	1.00 53.69 A
ATOM	34	CB	GLN	A	4	28.845	8.699	-15.580	1.00 54.21 A
ATOM	35	CG	GLN	A	4	29.861	8.974	-14.477	1.00 55.15 A
ATOM	36	CD	GLN	A	4	29.621	10.285	-13.732	1.00 55.56 A
ATOM	37	OE1	GLN	A	4	29.532	11.354	-14.343	1.00 56.19 A
ATOM	38	NE2	GLN	A	4	29.533	10.209	-12.403	1.00 55.66 A
ATOM	39	C	GLN	A	4	26.828	8.182	-14.212	1.00 53.19 A
ATOM	40	O	GLN	A	4	26.972	7.953	-13.008	1.00 53.10 A
ATOM	41	N	ILE	A	5	25.832	8.918	-14.705	1.00 52.58 A
ATOM	42	CA	ILE	A	5	24.817	9.523	-13.853	1.00 51.70 A
ATOM	43	CB	ILE	A	5	23.826	10.380	-14.687	1.00 51.71 A
ATOM	44	CG2	ILE	A	5	22.643	10.812	-13.831	1.00 51.41 A
ATOM	45	CG1	ILE	A	5	24.547	11.611	-15.246	1.00 51.48 A
ATOM	46	CD1	ILE	A	5	23.646	12.569	-16.017	1.00 51.33 A
ATOM	47	C	ILE	A	5	24.051	8.467	-13.060	1.00 51.26 A
ATOM	48	O	ILE	A	5	23.650	8.700	-11.920	1.00 51.09 A
ATOM	49	N	GLU	A	6	23.864	7.300	-13.662	1.00 50.54 A
ATOM	50	CA	GLU	A	6	23.146	6.214	-13.013	1.00 50.01 A
ATOM	51	CB	GLU	A	6	22.789	5.148	-14.043	1.00 50.43 A
ATOM	52	CG	GLU	A	6	22.141	5.721	-15.289	1.00 51.26 A
ATOM	53	CD	GLU	A	6	22.045	4.703	-16.400	1.00 51.68 A

Figure 7C

ATOM	54	OE1	GLU	A	6	23.016	3.931	-16.557	1.00	52.29	A
ATOM	55	OE2	GLU	A	6	21.019	4.682	-17.116	1.00	52.25	A
ATOM	56	C	GLU	A	6	23.995	5.606	-11.904	1.00	49.32	A
ATOM	57	O	GLU	A	6	23.475	5.210	-10.859	1.00	49.24	A
ATOM	58	N	ASP	A	7	25.302	5.527	-12.128	1.00	48.32	A
ATOM	59	CA	ASP	A	7	26.178	4.970	-11.113	1.00	47.23	A
ATOM	60	CB	ASP	A	7	27.543	4.626	-11.703	1.00	47.92	A
ATOM	61	CG	ASP	A	7	27.450	3.585	-12.788	1.00	46.33	A
ATOM	62	OD1	ASP	A	7	26.526	2.741	-12.729	1.00	48.43	A
ATOM	63	OD2	ASP	A	7	28.310	3.606	-13.690	1.00	48.94	A
ATOM	64	C	ASP	A	7	26.344	5.920	-9.926	1.00	46.09	A
ATOM	65	O	ASP	A	7	26.283	5.481	-8.773	1.00	45.71	A
ATOM	66	N	LYS	A	8	26.551	7.209	-10.201	1.00	44.57	A
ATOM	67	CA	LYS	A	8	26.703	8.195	-9.129	1.00	43.01	A
ATOM	68	CB	LYS	A	8	26.959	9.598	-9.708	1.00	43.49	A
ATOM	69	CG	LYS	A	8	25.895	10.076	-10.695	1.00	44.78	A
ATOM	70	CD	LYS	A	8	26.423	11.125	-11.702	1.00	45.38	A
ATOM	71	CE	LYS	A	8	26.698	12.490	-11.068	1.00	45.64	A
ATOM	72	NZ	LYS	A	8	27.153	13.499	-12.069	1.00	45.55	A
ATOM	73	C	LYS	A	8	25.413	8.171	-8.318	1.00	41.20	A
ATOM	74	O	LYS	A	8	25.419	8.346	-7.098	1.00	40.61	A
ATOM	75	N	ILE	A	9	24.302	7.935	-9.002	1.00	39.40	A
ATOM	76	CA	ILE	A	9	23.015	7.859	-8.333	1.00	37.29	A
ATOM	77	CB	ILE	A	9	21.872	7.859	-9.358	1.00	37.14	A
ATOM	78	CG2	ILE	A	9	20.600	7.251	-8.759	1.00	37.06	A
ATOM	79	CG1	ILE	A	9	21.631	9.303	-9.812	1.00	36.95	A
ATOM	80	CD1	ILE	A	9	20.801	9.440	-11.066	1.00	36.89	A
ATOM	81	C	ILE	A	9	22.927	6.638	-7.418	1.00	36.07	A
ATOM	82	O	ILE	A	9	22.450	6.756	-6.292	1.00	34.70	A
ATOM	83	N	GLU	A	10	23.389	5.478	-7.887	1.00	34.23	A
ATOM	84	CA	GLU	A	10	23.353	4.260	-7.074	1.00	33.04	A
ATOM	85	CB	GLU	A	10	23.884	3.013	-7.847	1.00	32.87	A
ATOM	86	CG	GLU	A	10	23.890	1.705	-6.991	1.00	33.10	A
ATOM	87	CD	GLU	A	10	24.287	0.417	-7.747	1.00	33.56	A
ATOM	88	OE1	GLU	A	10	24.327	0.442	-8.999	1.00	34.07	A
ATOM	89	OE2	GLU	A	10	24.542	-0.630	-7.084	1.00	32.41	A
ATOM	90	C	GLU	A	10	24.244	4.556	-5.878	1.00	32.53	A
ATOM	91	O	GLU	A	10	24.009	4.069	-4.779	1.00	32.14	A
ATOM	92	N	GLU	A	11	25.259	5.380	-6.100	1.00	31.82	A
ATOM	93	CA	GLU	A	11	26.165	5.731	-5.018	1.00	31.36	A
ATOM	94	CB	GLU	A	11	27.409	6.445	-5.536	1.00	33.18	A
ATOM	95	CG	GLU	A	11	28.358	6.833	-4.423	1.00	35.22	A
ATOM	96	CD	GLU	A	11	29.105	5.643	-3.822	1.00	36.93	A
ATOM	97	OE1	GLU	A	11	28.488	4.580	-3.575	1.00	38.03	A
ATOM	98	OE2	GLU	A	11	30.322	5.774	-3.579	1.00	38.85	A
ATOM	99	C	GLU	A	11	25.456	6.621	-3.998	1.00	30.15	A
ATOM	100	O	GLU	A	11	25.556	6.377	-2.798	1.00	28.89	A
ATOM	101	N	ILE	A	12	24.737	7.640	-4.471	1.00	29.09	A
ATOM	102	CA	ILE	A	12	24.017	8.533	-3.550	1.00	28.34	A
ATOM	103	CB	ILE	A	12	23.301	9.675	-4.325	1.00	28.74	A
ATOM	104	CG2	ILE	A	12	22.206	10.281	-3.501	1.00	28.70	A
ATOM	105	CG1	ILE	A	12	24.327	10.743	-4.701	1.00	28.84	A
ATOM	106	CD1	ILE	A	12	23.922	11.603	-5.890	1.00	29.69	A
ATOM	107	C	ILE	A	12	22.985	7.725	-2.761	1.00	27.83	A
ATOM	108	O	ILE	A	12	22.802	7.948	-1.560	1.00	26.46	A
ATOM	109	N	GLU	A	13	22.312	6.790	-3.423	1.00	27.40	A
ATOM	110	CA	GLU	A	13	21.313	5.965	-2.762	1.00	26.92	A
ATOM	111	CB	GLU	A	13	20.579	5.087	-3.805	1.00	28.34	A

Figure 7D

ATOM	112	CG	GLU	A	13	19.760	5.937	-4.810	1.00	29.72	A
ATOM	113	CD	GLU	A	13	19.080	5.118	-5.900	1.00	31.77	A
ATOM	114	OE1	GLU	A	13	19.671	4.107	-6.331	1.00	33.64	A
ATOM	115	OE2	GLU	A	13	17.960	5.495	-6.327	1.00	32.24	A
ATOM	116	C	GLU	A	13	21.975	5.110	-1.678	1.00	26.36	A
ATOM	117	O	GLU	A	13	21.411	4.912	-0.597	1.00	25.75	A
ATOM	118	N	SER	A	14	23.179	4.629	-1.950	1.00	26.17	A
ATOM	119	CA	SER	A	14	23.899	3.792	-0.999	1.00	26.31	A
ATOM	120	CB	SER	A	14	25.184	3.224	-1.625	1.00	26.71	A
ATOM	121	OG	SER	A	14	25.954	2.470	-0.695	1.00	30.07	A
ATOM	122	C	SER	A	14	24.246	4.626	0.221	1.00	25.81	A
ATOM	123	O	SER	A	14	24.079	4.149	1.339	1.00	25.13	A
ATOM	124	N	LYS	A	15	24.753	5.840	0.009	1.00	24.70	A
ATOM	125	CA	LYS	A	15	25.091	6.713	1.151	1.00	25.41	A
ATOM	126	CB	LYS	A	15	25.805	7.971	0.672	1.00	26.20	A
ATOM	127	CG	LYS	A	15	27.256	7.762	0.285	1.00	29.07	A
ATOM	128	CD	LYS	A	15	27.875	9.077	-0.220	1.00	30.97	A
ATOM	129	CE	LYS	A	15	29.328	8.914	-0.603	1.00	32.08	A
ATOM	130	NZ	LYS	A	15	29.547	7.749	-1.502	1.00	34.63	A
ATOM	131	C	LYS	A	15	23.824	7.102	1.938	1.00	24.45	A
ATOM	132	O	LYS	A	15	23.862	7.279	3.171	1.00	24.50	A
ATOM	133	N	GLN	A	16	22.708	7.254	1.247	1.00	24.12	A
ATOM	134	CA	GLN	A	16	21.450	7.586	1.904	1.00	23.82	A
ATOM	135	CB	GLN	A	16	20.396	7.815	0.834	1.00	25.71	A
ATOM	136	CG	GLN	A	16	19.229	8.643	1.232	1.00	29.64	A
ATOM	137	CD	GLN	A	16	18.543	9.230	0.004	1.00	32.26	A
ATOM	138	OE1	GLN	A	16	18.015	8.498	-0.817	1.00	34.89	A
ATOM	139	NE2	GLN	A	16	18.569	10.556	-0.135	1.00	32.74	A
ATOM	140	C	GLN	A	16	21.027	6.447	2.838	1.00	23.67	A
ATOM	141	O	GLN	A	16	20.584	6.681	3.979	1.00	22.84	A
ATOM	142	N	LYS	A	17	21.160	5.214	2.365	1.00	22.83	A
ATOM	143	CA	LYS	A	17	20.798	4.057	3.179	1.00	22.59	A
ATOM	144	CB	LYS	A	17	20.939	2.756	2.357	1.00	22.86	A
ATOM	145	CG	LYS	A	17	20.340	1.539	3.055	1.00	26.69	A
ATOM	146	CD	LYS	A	17	18.837	1.579	2.932	1.00	29.27	A
ATOM	147	CE	LYS	A	17	18.177	0.837	4.051	1.00	31.75	A
ATOM	148	NZ	LYS	A	17	16.686	0.870	3.940	1.00	34.25	A
ATOM	149	C	LYS	A	17	21.718	4.015	4.406	1.00	22.31	A
ATOM	150	O	LYS	A	17	21.261	3.747	5.515	1.00	21.02	A
ATOM	151	N	LYS	A	18	23.001	4.306	4.223	1.00	21.81	A
ATOM	152	CA	LYS	A	18	23.909	4.302	5.374	1.00	21.74	A
ATOM	153	CB	LYS	A	18	25.348	4.540	4.964	1.00	24.04	A
ATOM	154	CG	LYS	A	18	26.029	3.321	4.401	1.00	27.30	A
ATOM	155	CD	LYS	A	18	27.381	3.712	3.863	1.00	29.23	A
ATOM	156	CE	LYS	A	18	27.972	2.592	3.025	1.00	30.50	A
ATOM	157	NZ	LYS	A	18	29.290	3.010	2.472	1.00	33.57	A
ATOM	158	C	LYS	A	18	23.500	5.376	6.378	1.00	20.62	A
ATOM	159	O	LYS	A	18	23.565	5.138	7.577	1.00	19.85	A
ATOM	160	N	ILE	A	19	23.062	6.531	5.887	1.00	19.99	A
ATOM	161	CA	ILE	A	19	22.655	7.636	6.762	1.00	19.98	A
ATOM	162	CB	ILE	A	19	22.406	8.926	5.914	1.00	20.09	A
ATOM	163	CG2	ILE	A	19	21.554	9.944	6.682	1.00	20.80	A
ATOM	164	CG1	ILE	A	19	23.756	9.499	5.464	1.00	21.49	A
ATOM	165	CD1	ILE	A	19	23.669	10.495	4.296	1.00	21.18	A
ATOM	166	C	ILE	A	19	21.400	7.221	7.517	1.00	20.44	A
ATOM	167	O	ILE	A	19	21.282	7.452	8.735	1.00	20.23	A
ATOM	168	N	GLU	A	20	20.459	6.569	6.836	1.00	20.24	A
ATOM	169	CA	GLU	A	20	19.230	6.149	7.503	1.00	20.43	A

Figure 7E

ATOM	170	CB	GLU	A	20	18.223	5.608	6.484	1.00	22.94	A
ATOM	171	CG	GLU	A	20	17.766	6.671	5.499	1.00	25.51	A
ATOM	172	CD	GLU	A	20	16.926	6.108	4.378	1.00	29.04	A
ATOM	173	OE1	GLU	A	20	16.961	4.873	4.177	1.00	30.40	A
ATOM	174	OE2	GLU	A	20	16.243	6.901	3.691	1.00	30.73	A
ATOM	175	C	GLU	A	20	19.533	5.109	8.576	1.00	20.88	A
ATOM	176	O	GLU	A	20	18.917	5.127	9.645	1.00	20.23	A
ATOM	177	N	ASN	A	21	20.478	4.220	8.321	1.00	20.53	A
ATOM	178	CA	ASN	A	21	20.820	3.212	9.328	1.00	21.87	A
ATOM	179	CB	ASN	A	21	21.694	2.117	8.720	1.00	24.15	A
ATOM	180	CG	ASN	A	21	20.875	1.155	7.872	1.00	25.28	A
ATOM	181	OD1	ASN	A	21	19.676	0.980	8.099	1.00	28.26	A
ATOM	182	ND2	ASN	A	21	21.505	0.549	6.870	1.00	26.78	A
ATOM	183	C	ASN	A	21	21.500	3.854	10.527	1.00	21.75	A
ATOM	184	O	ASN	A	21	21.269	3.444	11.674	1.00	21.80	A
ATOM	185	N	GLU	A	22	22.335	4.853	10.274	1.00	20.99	A
ATOM	186	CA	GLU	A	22	23.007	5.548	11.369	1.00	20.36	A
ATOM	187	CB	GLU	A	22	24.059	6.516	10.825	1.00	22.89	A
ATOM	188	CG	GLU	A	22	24.914	7.169	11.901	1.00	25.86	A
ATOM	189	CD	GLU	A	22	25.515	6.170	12.882	1.00	27.97	A
ATOM	190	OE1	GLU	A	22	26.121	5.158	12.444	1.00	30.05	A
ATOM	191	OE2	GLU	A	22	25.376	6.411	14.118	1.00	31.29	A
ATOM	192	C	GLU	A	22	21.952	6.294	12.187	1.00	19.79	A
ATOM	193	O	GLU	A	22	21.988	6.264	13.445	1.00	18.87	A
ATOM	194	N	ILE	A	23	21.003	6.951	11.518	1.00	18.92	A
ATOM	195	CA	ILE	A	23	19.955	7.670	12.254	1.00	18.60	A
ATOM	196	CB	ILE	A	23	19.012	8.388	11.244	1.00	18.79	A
ATOM	197	CG2	ILE	A	23	17.672	8.764	11.880	1.00	20.11	A
ATOM	198	CG1	ILE	A	23	19.739	9.598	10.701	1.00	20.45	A
ATOM	199	CD1	ILE	A	23	19.060	10.223	9.539	1.00	22.51	A
ATOM	200	C	ILE	A	23	19.163	6.687	13.118	1.00	19.09	A
ATOM	201	O	ILE	A	23	18.807	7.006	14.260	1.00	18.74	A
ATOM	202	N	ALA	A	24	18.903	5.479	12.617	1.00	18.44	A
ATOM	203	CA	ALA	A	24	18.153	4.517	13.420	1.00	18.86	A
ATOM	204	CB	ALA	A	24	17.824	3.257	12.573	1.00	19.39	A
ATOM	205	C	ALA	A	24	18.947	4.136	14.665	1.00	18.66	A
ATOM	206	O	ALA	A	24	18.343	3.966	15.757	1.00	19.32	A
ATOM	207	N	ARG	A	25	20.272	4.028	14.548	1.00	18.57	A
ATOM	208	CA	ARG	A	25	21.111	3.667	15.709	1.00	19.19	A
ATOM	209	CB	ARG	A	25	22.552	3.343	15.287	1.00	20.85	A
ATOM	210	CG	ARG	A	25	22.674	1.959	14.627	1.00	23.87	A
ATOM	211	CD	ARG	A	25	24.108	1.536	14.429	1.00	25.32	A
ATOM	212	NE	ARG	A	25	24.759	2.294	13.376	1.00	27.13	A
ATOM	213	CZ	ARG	A	25	24.672	2.019	12.075	1.00	27.60	A
ATOM	214	NH1	ARG	A	25	23.955	0.979	11.641	1.00	28.92	A
ATOM	215	NH2	ARG	A	25	25.296	2.806	11.214	1.00	27.79	A
ATOM	216	C	ARG	A	25	21.083	4.819	16.722	1.00	18.69	A
ATOM	217	O	ARG	A	25	20.942	4.592	17.940	1.00	17.93	A
ATOM	218	N	ILE	A	26	21.201	6.041	16.221	1.00	17.83	A
ATOM	219	CA	ILE	A	26	21.184	7.222	17.080	1.00	16.94	A
ATOM	220	CB	ILE	A	26	21.369	8.479	16.225	1.00	17.99	A
ATOM	221	CG2	ILE	A	26	20.943	9.741	17.006	1.00	19.34	A
ATOM	222	CG1	ILE	A	26	22.821	8.537	15.796	1.00	19.88	A
ATOM	223	CD1	ILE	A	26	23.144	9.587	14.721	1.00	21.83	A
ATOM	224	C	ILE	A	26	19.876	7.301	17.857	1.00	18.02	A
ATOM	225	O	ILE	A	26	19.875	7.580	19.055	1.00	17.73	A
ATOM	226	N	LYS	A	27	18.752	7.069	17.191	1.00	17.60	A
ATOM	227	CA	LYS	A	27	17.450	7.137	17.853	1.00	17.90	A

Figure 7F

ATOM	228	CB	LYS	A	27	16.330	6.994	16.805	1.00	19.01	A
ATOM	229	CG	LYS	A	27	16.266	8.210	15.876	1.00	22.27	A
ATOM	230	CD	LYS	A	27	15.275	7.984	14.711	1.00	24.03	A
ATOM	231	CE	LYS	A	27	13.860	7.664	15.161	1.00	24.41	A
ATOM	232	NZ	LYS	A	27	13.173	8.848	15.714	1.00	27.04	A
ATOM	233	C	LYS	A	27	17.326	6.097	18.969	1.00	18.17	A
ATOM	234	O	LYS	A	27	16.767	6.388	20.013	1.00	18.33	A
ATOM	235	N	LYS	A	28	17.871	4.896	18.775	1.00	17.00	A
ATOM	236	CA	LYS	A	28	17.788	3.867	19.790	1.00	17.21	A
ATOM	237	CB	LYS	A	28	18.244	2.503	19.223	1.00	18.92	A
ATOM	238	CG	LYS	A	28	17.288	1.982	18.164	1.00	24.56	A
ATOM	239	CD	LYS	A	28	17.833	0.732	17.464	1.00	26.88	A
ATOM	240	CE	LYS	A	28	16.950	0.371	16.260	1.00	28.84	A
ATOM	241	NZ	LYS	A	28	17.284	-0.938	15.592	1.00	31.36	A
ATOM	242	C	LYS	A	28	18.618	4.257	21.016	1.00	17.36	A
ATOM	243	O	LYS	A	28	18.169	4.066	22.165	1.00	17.54	A
ATOM	244	N	LEU	A	29	19.794	4.835	20.793	1.00	16.84	A
ATOM	245	CA	LEU	A	29	20.642	5.234	21.912	1.00	16.41	A
ATOM	246	CB	LEU	A	29	22.077	5.529	21.453	1.00	16.26	A
ATOM	247	CG	LEU	A	29	23.050	6.048	22.515	1.00	16.76	A
ATOM	248	CD1	LEU	A	29	23.062	5.096	23.701	1.00	16.47	A
ATOM	249	CD2	LEU	A	29	24.450	6.201	21.885	1.00	17.67	A
ATOM	250	C	LEU	A	29	20.023	6.429	22.606	1.00	16.92	A
ATOM	251	O	LEU	A	29	20.027	6.503	23.859	1.00	16.36	A
ATOM	252	N	LEU	A	30	19.447	7.343	21.820	1.00	15.57	A
ATOM	253	CA	LEU	A	30	18.818	8.519	22.424	1.00	15.77	A
ATOM	254	CB	LEU	A	30	18.401	9.501	21.298	1.00	15.65	A
ATOM	255	CG	LEU	A	30	17.717	10.780	21.696	1.00	17.55	A
ATOM	256	CD1	LEU	A	30	18.557	11.504	22.722	1.00	16.71	A
ATOM	257	CD2	LEU	A	30	17.552	11.602	20.399	1.00	18.10	A
ATOM	258	C	LEU	A	30	17.659	8.067	23.288	1.00	16.42	A
ATOM	259	O	LEU	A	30	17.466	8.604	24.399	1.00	17.55	A
ATOM	260	N	GLN	A	31	16.903	7.053	22.862	1.00	16.79	A
ATOM	261	CA	GLN	A	31	15.816	6.564	23.692	1.00	18.13	A
ATOM	262	CB	GLN	A	31	14.945	5.593	22.886	1.00	21.45	A
ATOM	263	CG	GLN	A	31	14.119	6.358	21.834	1.00	24.92	A
ATOM	264	CD	GLN	A	31	13.196	7.437	22.424	1.00	26.81	A
ATOM	265	OE1	GLN	A	31	12.913	8.459	21.786	1.00	28.75	A
ATOM	266	NE2	GLN	A	31	12.713	7.207	23.648	1.00	29.86	A
ATOM	267	C	GLN	A	31	16.319	5.958	25.008	1.00	17.24	A
ATOM	268	O	GLN	A	31	15.655	6.092	26.038	1.00	17.79	A
ATOM	269	N	LEU	A	32	17.494	5.307	24.987	1.00	15.77	A
ATOM	270	CA	LEU	A	32	18.070	4.755	26.209	1.00	14.63	A
ATOM	271	CB	LEU	A	32	19.314	3.932	25.911	1.00	16.13	A
ATOM	272	CG	LEU	A	32	19.015	2.574	25.275	1.00	18.58	A
ATOM	273	CD1	LEU	A	32	20.291	1.961	24.770	1.00	20.70	A
ATOM	274	CD2	LEU	A	32	18.337	1.698	26.315	1.00	22.17	A
ATOM	275	C	LEU	A	32	18.449	5.895	27.140	1.00	13.68	A
ATOM	276	O	LEU	A	32	18.258	5.774	28.357	1.00	13.31	A
ATOM	277	N	THR	A	33	18.980	6.991	26.600	1.00	13.42	A
ATOM	278	CA	THR	A	33	19.348	8.081	27.500	1.00	12.96	A
ATOM	279	CB	THR	A	33	20.236	9.134	26.820	1.00	13.48	A
ATOM	280	OG1	THR	A	33	19.530	9.745	25.733	1.00	15.60	A
ATOM	281	CG2	THR	A	33	21.567	8.508	26.358	1.00	15.01	A
ATOM	282	C	THR	A	33	18.124	8.742	28.117	1.00	13.65	A
ATOM	283	O	THR	A	33	18.159	9.169	29.285	1.00	12.67	A
ATOM	284	N	VAL	A	34	17.038	8.838	27.345	1.00	13.20	A
ATOM	285	CA	VAL	A	34	15.804	9.410	27.863	1.00	13.88	A

Figure 7C



ATOM	286	CB	VAL	A	34	14.708	9.498	26.773	1.00	14.31	A
ATOM	287	CG1	VAL	A	34	13.380	9.811	27.382	1.00	15.35	A
ATOM	288	CG2	VAL	A	34	15.096	10.517	25.710	1.00	15.04	A
ATOM	289	C	VAL	A	34	15.326	8.526	29.041	1.00	12.55	A
ATOM	290	O	VAL	A	34	14.997	9.016	30.131	1.00	13.43	A
ATOM	291	N	TRP	A	35	15.354	7.210	28.857	1.00	13.04	A
ATOM	292	CA	TRP	A	35	14.946	6.289	29.908	1.00	13.11	A
ATOM	293	CB	TRP	A	35	14.988	4.861	29.319	1.00	14.19	A
ATOM	294	CG	TRP	A	35	14.672	3.785	30.334	1.00	15.43	A
ATOM	295	CD2	TRP	A	35	15.610	3.101	31.191	1.00	15.26	A
ATOM	296	CE2	TRP	A	35	14.860	2.165	31.963	1.00	15.57	A
ATOM	297	CE3	TRP	A	35	16.990	3.196	31.393	1.00	15.49	A
ATOM	298	CD1	TRP	A	35	13.454	3.258	30.609	1.00	17.15	A
ATOM	299	NE1	TRP	A	35	13.553	2.281	31.572	1.00	17.80	A
ATOM	300	CZ2	TRP	A	35	15.459	1.324	32.905	1.00	15.31	A
ATOM	301	CZ3	TRP	A	35	17.600	2.355	32.349	1.00	16.17	A
ATOM	302	CH2	TRP	A	35	16.815	1.437	33.090	1.00	14.74	A
ATOM	303	C	TRP	A	35	15.869	6.429	31.141	1.00	13.13	A
ATOM	304	O	TRP	A	35	15.418	6.409	32.278	1.00	12.76	A
ATOM	305	N	GLY	A	36	17.176	6.556	30.893	1.00	12.50	A
ATOM	306	CA	GLY	A	36	18.118	6.668	31.998	1.00	12.50	A
ATOM	307	C	GLY	A	36	17.887	7.936	32.817	1.00	11.58	A
ATOM	308	O	GLY	A	36	17.917	7.875	34.042	1.00	11.70	A
ATOM	309	N	ILE	A	37	17.656	9.084	32.174	1.00	11.85	A
ATOM	310	CA	ILE	A	37	17.383	10.303	32.884	1.00	11.18	A
ATOM	311	CB	ILE	A	37	17.262	11.439	31.882	1.00	11.22	A
ATOM	312	CG2	ILE	A	37	16.680	12.660	32.600	1.00	13.25	A
ATOM	313	CG1	ILE	A	37	18.636	11.739	31.281	1.00	12.70	A
ATOM	314	CD1	ILE	A	37	18.571	12.560	29.955	1.00	13.00	A
ATOM	315	C	ILE	A	37	16.082	10.105	33.703	1.00	11.99	A
ATOM	316	O	ILE	A	37	16.026	10.526	34.860	1.00	12.24	A
ATOM	317	N	LYS	A	38	15.069	9.465	33.094	1.00	11.84	A
ATOM	318	CA	LYS	A	38	13.825	9.215	33.809	1.00	13.62	A
ATOM	319	CB	LYS	A	38	12.840	8.512	32.861	1.00	15.00	A
ATOM	320	CG	LYS	A	38	11.429	8.437	33.369	1.00	17.76	A
ATOM	321	CD	LYS	A	38	10.545	7.835	32.247	1.00	20.78	A
ATOM	322	CE	LYS	A	38	9.046	7.955	32.600	1.00	25.34	A
ATOM	323	NZ	LYS	A	38	8.721	7.069	33.722	1.00	29.03	A
ATOM	324	C	LYS	A	38	14.060	8.399	35.083	1.00	12.64	A
ATOM	325	O	LYS	A	38	13.490	8.724	36.163	1.00	12.58	A
ATOM	326	N	GLN	A	39	14.916	7.371	35.001	1.00	11.99	A
ATOM	327	CA	GLN	A	39	15.176	6.573	36.189	1.00	11.84	A
ATOM	328	CB	GLN	A	39	16.049	5.339	35.900	1.00	12.90	A
ATOM	329	CG	GLN	A	39	15.580	4.440	34.757	1.00	14.71	A
ATOM	330	CD	GLN	A	39	14.118	4.213	34.747	1.00	17.73	A
ATOM	331	OE1	GLN	A	39	13.596	3.581	35.669	1.00	22.45	A
ATOM	332	NE2	GLN	A	39	13.420	4.701	33.701	1.00	20.02	A
ATOM	333	C	GLN	A	39	15.907	7.372	37.259	1.00	12.24	A
ATOM	334	O	GLN	A	39	15.601	7.271	38.453	1.00	12.42	A
ATOM	335	N	LEU	A	40	16.883	8.195	36.854	1.00	10.89	A
ATOM	336	CA	LEU	A	40	17.632	8.980	37.853	1.00	11.44	A
ATOM	337	CB	LEU	A	40	18.860	9.648	37.198	1.00	12.26	A
ATOM	338	CG	LEU	A	40	19.827	8.591	36.635	1.00	12.85	A
ATOM	339	CD1	LEU	A	40	21.007	9.367	36.066	1.00	16.06	A
ATOM	340	CD2	LEU	A	40	20.293	7.526	37.650	1.00	17.91	A
ATOM	341	C	LEU	A	40	16.763	10.046	38.497	1.00	10.71	A
ATOM	342	O	LEU	A	40	16.848	10.258	39.701	1.00	11.30	A
ATOM	343	N	GLN	A	41	15.911	10.692	37.704	1.00	11.62	A

Figure 7H

ATOM	344	CA	GLN	A	41	15.038	11.695	38.322	1.00	11.12	A
ATOM	345	CB	GLN	A	41	14.241	12.447	37.257	1.00	11.92	A
ATOM	346	CG	GLN	A	41	13.250	13.381	37.845	1.00	11.53	A
ATOM	347	CD	GLN	A	41	12.280	13.933	36.838	1.00	12.64	A
ATOM	348	OE1	GLN	A	41	11.814	13.226	35.962	1.00	13.16	A
ATOM	349	NE2	GLN	A	41	11.972	15.220	36.973	1.00	13.67	A
ATOM	350	C	GLN	A	41	14.082	11.031	39.332	1.00	10.98	A
ATOM	351	O	GLN	A	41	13.883	11.585	40.404	1.00	12.39	A
ATOM	352	N	ALA	A	42	13.571	9.845	38.994	1.00	12.53	A
ATOM	353	CA	ALA	A	42	12.642	9.185	39.928	1.00	12.08	A
ATOM	354	CB	ALA	A	42	12.035	7.954	39.295	1.00	13.83	A
ATOM	355	C	ALA	A	42	13.383	8.856	41.218	1.00	14.57	A
ATOM	356	O	ALA	A	42	12.820	8.975	42.296	1.00	15.73	A
ATOM	357	N	ARG	A	43	14.647	8.446	41.147	1.00	13.64	A
ATOM	358	CA	ARG	A	43	15.412	8.150	42.327	1.00	16.22	A
ATOM	359	CB	ARG	A	43	16.772	7.626	41.852	1.00	18.06	A
ATOM	360	CG	ARG	A	43	17.706	7.309	42.895	1.00	22.64	A
ATOM	361	CD	ARG	A	43	17.232	6.108	43.679	1.00	25.20	A
ATOM	362	NE	ARG	A	43	18.302	5.922	44.577	1.00	27.65	A
ATOM	363	CZ	ARG	A	43	18.943	4.798	44.758	1.00	20.75	A
ATOM	364	NH1	ARG	A	43	18.607	3.666	44.107	1.00	24.75	A
ATOM	365	NH2	ARG	A	43	19.983	4.899	45.516	1.00	23.93	A
ATOM	366	C	ARG	A	43	15.606	9.411	43.196	1.00	15.07	A
ATOM	367	O	ARG	A	43	15.441	9.372	44.435	1.00	17.46	A
ATOM	368	N	ILE	A	44	15.930	10.529	42.553	1.00	14.44	A
ATOM	369	CA	ILE	A	44	16.181	11.794	43.242	1.00	14.63	A
ATOM	370	CB	ILE	A	44	16.801	12.854	42.280	1.00	15.70	A
ATOM	371	CG2	ILE	A	44	16.817	14.226	42.941	1.00	16.89	A
ATOM	372	CG1	ILE	A	44	18.236	12.422	41.940	1.00	16.08	A
ATOM	373	CD1	ILE	A	44	18.765	13.127	40.739	1.00	19.48	A
ATOM	374	C	ILE	A	44	14.906	12.326	43.887	1.00	16.35	A
ATOM	375	O	ILE	A	44	14.984	12.862	44.991	1.00	19.28	A
ATOM	376	N	LEU	A	45	13.747	12.150	43.258	1.00	15.72	A
ATOM	377	CA	LEU	A	45	12.515	12.682	43.883	1.00	15.80	A
ATOM	378	CB	LEU	A	45	11.505	13.032	42.801	1.00	15.66	A
ATOM	379	CG	LEU	A	45	11.867	14.181	41.878	1.00	15.35	A
ATOM	380	CD1	LEU	A	45	10.793	14.298	40.823	1.00	17.27	A
ATOM	381	CD2	LEU	A	45	11.954	15.485	42.701	1.00	18.49	A
ATOM	382	C	LEU	A	45	11.903	11.710	44.867	1.00	18.22	A
ATOM	383	O	LEU	A	45	11.053	12.187	45.658	1.00	19.14	A
ATOM	384	NT	LEU	A	45	12.258	10.488	44.884	1.00	20.39	A
ATOM	385	CA	ACE	D	0	10.275	-0.794	28.942	1.00	41.14	B
ATOM	386	C	ACE	D	0	11.674	-0.285	28.785	1.00	40.52	B
ATOM	387	O	ACE	D	0	11.905	0.677	28.016	1.00	41.12	B
ATOM	388	N	DLY	D	1	12.631	-0.899	29.487	1.00	39.74	B
ATOM	389	CA	DLY	D	1	13.997	-0.423	29.356	1.00	37.31	B
ATOM	390	C	DLY	D	1	15.200	-1.051	30.044	1.00	35.38	B
ATOM	391	O	DLY	D	1	15.133	-2.044	30.785	1.00	35.49	B
ATOM	392	N	DLA	D	2	16.332	-0.424	29.752	1.00	33.19	B
ATOM	393	CA	DLA	D	2	17.639	-0.797	30.279	1.00	31.99	B
ATOM	394	CB	DLA	D	2	18.688	0.196	29.762	1.00	31.34	B
ATOM	395	C	DLA	D	2	18.026	-2.217	29.871	1.00	31.71	B
ATOM	396	O	DLA	D	2	18.611	-2.982	30.647	1.00	31.67	B
ATOM	397	N	DCS	D	3	17.699	-2.577	28.640	1.00	30.76	B
ATOM	398	CA	DCS	D	3	18.061	-3.892	28.159	1.00	31.11	B
ATOM	399	C	DCS	D	3	17.104	-4.987	28.618	1.00	31.69	B
ATOM	400	O	DCS	D	3	17.531	-6.020	29.111	1.00	31.85	B
ATOM	401	CB	DCS	D	3	18.128	-3.876	26.638	1.00	30.00	B

Figure 7I

ATOM	402	SG	DCS	D	3	19.502	-2.991	25.840	1.00	30.98	B
ATOM	403	N	DLU	D	4	15.813	-4.736	28.474	1.00	31.68	B
ATOM	404	CA	DLU	D	4	14.782	-5.702	28.834	1.00	32.07	B
ATOM	405	CB	DLU	D	4	13.397	-5.090	28.574	1.00	33.43	B
ATOM	406	CG	DLU	D	4	13.060	-4.844	27.093	1.00	35.53	B
ATOM	407	CD	DLU	D	4	13.663	-3.568	26.500	1.00	36.29	B
ATOM	408	OE1	DLU	D	4	14.422	-2.859	27.182	1.00	37.11	B
ATOM	409	OE2	DLU	D	4	13.367	-3.264	25.323	1.00	37.45	B
ATOM	410	C	DLU	D	4	14.875	-6.180	30.276	1.00	31.86	B
ATOM	411	O	DLU	D	4	14.832	-7.381	30.553	1.00	32.10	B
ATOM	412	N	DLA	D	5	15.022	-5.237	31.196	1.00	30.98	B
ATOM	413	CA	DLA	D	5	15.098	-5.566	32.611	1.00	30.61	B
ATOM	414	CB	DLA	D	5	14.984	-4.296	33.406	1.00	30.83	B
ATOM	415	C	DLA	D	5	16.362	-6.340	33.008	1.00	30.19	B
ATOM	416	O	DLA	D	5	16.387	-7.044	34.027	1.00	30.60	B
ATOM	417	N	DRG	D	6	17.418	-6.202	32.216	1.00	29.09	B
ATOM	418	CA	DRG	D	6	18.673	-6.893	32.489	1.00	28.71	B
ATOM	419	CB	DRG	D	6	18.480	-8.408	32.369	1.00	31.46	B
ATOM	420	CG	DRG	D	6	18.169	-8.847	30.969	1.00	34.88	B
ATOM	421	CD	DRG	D	6	19.397	-8.762	30.070	1.00	37.42	B
ATOM	422	NE	DRG	D	6	19.715	-7.408	29.607	1.00	40.28	B
ATOM	423	CZ	DRG	D	6	20.121	-7.134	28.370	1.00	40.89	B
ATOM	424	NH1	DRG	D	6	20.248	-8.118	27.481	1.00	42.76	B
ATOM	425	NH2	DRG	D	6	20.409	-5.891	28.015	1.00	42.55	B
ATOM	426	C	DRG	D	6	19.313	-6.582	33.833	1.00	27.29	B
ATOM	427	O	DRG	D	6	19.994	-7.423	34.421	1.00	27.43	B
ATOM	428	N	DIS	D	7	19.100	-5.379	34.342	1.00	24.49	B
ATOM	429	CA	DIS	D	7	19.731	-5.018	35.624	1.00	22.04	B
ATOM	430	CB	DIS	D	7	18.970	-3.888	36.284	1.00	22.68	B
ATOM	431	CG	DIS	D	7	17.655	-4.321	36.854	1.00	22.88	B
ATOM	432	CD2	DIS	D	7	17.178	-5.567	37.104	1.00	24.08	B
ATOM	433	ND1	DIS	D	7	16.650	-3.445	37.187	1.00	25.78	B
ATOM	434	CE1	DIS	D	7	15.595	-4.134	37.608	1.00	26.45	B
ATOM	435	NE2	DIS	D	7	15.894	-5.419	37.562	1.00	25.11	B
ATOM	436	C	DIS	D	7	21.156	-4.636	35.329	1.00	21.84	B
ATOM	437	O	DIS	D	7	21.412	-3.743	34.536	1.00	20.32	B
ATOM	438	N	DRG	D	8	22.091	-5.298	36.003	1.00	20.33	B
ATOM	439	CA	DRG	D	8	23.494	-5.122	35.778	1.00	19.80	B
ATOM	440	CB	DRG	D	8	24.284	-5.994	36.755	1.00	20.87	B
ATOM	441	CG	DRG	D	8	24.175	-7.428	36.459	1.00	26.97	B
ATOM	442	CD	DRG	D	8	24.743	-8.207	37.631	1.00	29.07	B
ATOM	443	NE	DRG	D	8	24.581	-9.603	37.325	1.00	31.54	B
ATOM	444	CZ	DRG	D	8	25.258	-10.189	36.352	1.00	31.94	B
ATOM	445	NH1	DRG	D	8	26.139	-9.485	35.658	1.00	33.88	B
ATOM	446	NH2	DRG	D	8	24.987	-11.432	36.027	1.00	33.88	B
ATOM	447	C	DRG	D	8	23.985	-3.711	35.873	1.00	17.95	B
ATOM	448	O	DRG	D	8	24.856	-3.361	35.124	1.00	17.42	B
ATOM	449	N	DLU	D	9	23.407	-2.934	36.783	1.00	16.93	B
ATOM	450	CA	DLU	D	9	23.900	-1.578	36.951	1.00	15.49	B
ATOM	451	CB	DLU	D	9	23.358	-0.954	38.261	1.00	16.03	B
ATOM	452	CG	DLU	D	9	21.876	-0.652	38.323	1.00	16.75	B
ATOM	453	CD	DLU	D	9	20.996	-1.816	38.786	1.00	16.82	B
ATOM	454	OE1	DLU	D	9	21.407	-2.982	38.584	1.00	19.63	B
ATOM	455	OE2	DLU	D	9	19.933	-1.498	39.310	1.00	20.12	B
ATOM	456	C	DLU	D	9	23.601	-0.717	35.747	1.00	15.97	B
ATOM	457	O	DLU	D	9	24.142	0.383	35.655	1.00	15.24	B
ATOM	458	N	DRP	D	10	22.747	-1.186	34.844	1.00	15.66	B
ATOM	459	CA	DRP	D	10	22.462	-0.435	33.611	1.00	15.31	B

Figure 7J

ATOM	460	CB	DRP	D	10	20.960	-0.187	33.420	1.00	16.05	B
ATOM	461	CG	DRP	D	10	20.354	0.791	34.410	1.00	15.28	B
ATOM	462	CD2	DRP	D	10	20.504	2.200	34.384	1.00	15.28	B
ATOM	463	CE2	DRP	D	10	19.734	2.730	35.424	1.00	15.74	B
ATOM	464	CE3	DRP	D	10	21.237	3.075	33.563	1.00	15.47	B
ATOM	465	CD1	DRP	D	10	19.504	0.512	35.449	1.00	16.40	B
ATOM	466	NE1	DRP	D	10	19.122	1.676	36.073	1.00	17.22	B
ATOM	467	CZ2	DRP	D	10	19.650	4.107	35.666	1.00	15.81	B
ATOM	468	CZ3	DRP	D	10	21.174	4.444	33.805	1.00	14.93	B
ATOM	469	CH2	DRP	D	10	20.382	4.935	34.850	1.00	15.26	B
ATOM	470	C	DRP	D	10	23.000	-1.140	32.376	1.00	17.32	B
ATOM	471	O	DRP	D	10	22.790	-0.682	31.244	1.00	16.59	B
ATOM	472	N	DLA	D	11	23.744	-2.227	32.572	1.00	17.72	B
ATOM	473	CA	DLA	D	11	24.253	-2.940	31.407	1.00	18.88	B
ATOM	474	CB	DLA	D	11	25.034	-4.168	31.867	1.00	20.11	B
ATOM	475	C	DLA	D	11	25.126	-2.074	30.501	1.00	18.95	B
ATOM	476	O	DLA	D	11	25.078	-2.221	29.267	1.00	21.13	B
ATOM	477	N	DRP	D	12	25.884	-1.142	31.084	1.00	17.86	B
ATOM	478	CA	DRP	D	12	26.759	-0.275	30.317	1.00	17.72	B
ATOM	479	CB	DRP	D	12	27.586	0.645	31.239	1.00	18.43	B
ATOM	480	CG	DRP	D	12	26.725	1.588	32.059	1.00	16.68	B
ATOM	481	CD2	DRP	D	12	26.285	2.900	31.676	1.00	16.49	B
ATOM	482	CE2	DRP	D	12	25.459	3.371	32.706	1.00	15.68	B
ATOM	483	CE3	DRP	D	12	26.519	3.714	30.561	1.00	17.14	B
ATOM	484	CD1	DRP	D	12	26.177	1.335	33.256	1.00	15.60	B
ATOM	485	NE1	DRP	D	12	25.402	2.400	33.668	1.00	15.74	B
ATOM	486	CZ2	DRP	D	12	24.842	4.628	32.664	1.00	15.78	B
ATOM	487	CZ3	DRP	D	12	25.904	4.977	30.525	1.00	17.42	B
ATOM	488	CH2	DRP	D	12	25.090	5.406	31.550	1.00	16.81	B
ATOM	489	C	DRP	D	12	25.913	0.577	29.346	1.00	18.81	B
ATOM	490	O	DRP	D	12	26.347	0.870	28.231	1.00	20.05	B
ATOM	491	N	DEU	D	13	24.740	1.020	29.790	1.00	17.43	B
ATOM	492	CA	DEU	D	13	23.915	1.866	28.926	1.00	17.59	B
ATOM	493	CB	DEU	D	13	22.883	2.647	29.756	1.00	15.97	B
ATOM	494	CG	DEU	D	13	21.857	3.489	28.971	1.00	15.31	B
ATOM	495	CD1	DEU	D	13	22.559	4.585	28.204	1.00	16.99	B
ATOM	496	CD2	DEU	D	13	20.886	4.105	29.938	1.00	16.07	B
ATOM	497	C	DEU	D	13	23.265	1.011	27.847	1.00	19.32	B
ATOM	498	O	DEU	D	13	23.224	1.429	26.702	1.00	20.12	B
ATOM	499	N	DCS	D	14	22.775	-0.180	28.199	1.00	20.93	B
ATOM	500	CA	DCS	D	14	22.190	-1.046	27.196	1.00	22.79	B
ATOM	501	C	DCS	D	14	23.272	-1.329	26.124	1.00	22.54	B
ATOM	502	O	DCS	D	14	22.963	-1.318	24.916	1.00	23.67	B
ATOM	503	CB	DCS	D	14	21.675	-2.319	27.874	1.00	23.47	B
ATOM	504	SG	DCS	D	14	21.216	-3.669	26.732	1.00	27.91	B
ATOM	505	N	DLA	D	15	24.514	-1.568	26.533	1.00	22.47	B
ATOM	506	CA	DLA	D	15	25.627	-1.857	25.614	1.00	23.31	B
ATOM	507	CB	DLA	D	15	26.868	-2.302	26.401	1.00	24.09	B
ATOM	508	C	DLA	D	15	25.987	-0.672	24.717	1.00	24.16	B
ATOM	509	O	DLA	D	15	26.511	-0.844	23.614	1.00	25.93	B
ATOM	510	N	DLA	D	16	25.723	0.544	25.192	1.00	22.60	B
ATOM	511	CA	DLA	D	16	26.017	1.743	24.400	1.00	22.10	B
ATOM	512	CB	DLA	D	16	26.006	2.985	25.314	1.00	22.02	B
ATOM	513	C	DLA	D	16	24.995	1.932	23.278	1.00	21.95	B
ATOM	514	O	DLA	D	16	25.355	2.570	22.256	1.00	22.36	B
ATOM	515	NT	DLA	D	16	23.843	1.460	23.410	1.00	23.47	B
ATOM	516	CL-1	CL	I	1	20.914	12.075	1.899	1.00	45.04	I
ATOM	517	OH2	WAT	W	1	23.911	6.454	-21.684	1.00	53.50	W

Figure 7K

ATOM	518	OH2	WAT	W	2	30.822	2.444	-19.357	1.00	52.17	W
ATOM	519	OH2	WAT	W	3	30.369	13.971	-17.693	1.00	37.33	W
ATOM	520	OH2	WAT	W	4	27.699	12.875	-16.588	1.00	46.63	W
ATOM	521	OH2	WAT	W	5	23.417	1.727	-13.168	1.00	48.41	W
ATOM	522	OH2	WAT	W	6	24.012	1.401	-16.007	1.00	58.65	W
ATOM	523	OH2	WAT	W	7	16.572	3.069	-7.418	1.00	36.12	W
ATOM	524	OH2	WAT	W	8	32.381	11.028	-8.334	1.00	55.01	W
ATOM	525	OH2	WAT	W	9	33.753	7.275	-10.261	1.00	53.14	W
ATOM	526	OH2	WAT	W	10	20.318	-0.862	-12.067	1.00	28.89	W
ATOM	527	OH2	WAT	W	11	26.434	1.459	-10.129	1.00	43.04	W
ATOM	528	OH2	WAT	W	12	27.878	0.323	-12.146	1.00	55.95	W
ATOM	529	OH2	WAT	W	13	31.427	0.259	-10.741	1.00	52.47	W
ATOM	530	OH2	WAT	W	14	29.889	8.411	-6.889	1.00	56.49	W
ATOM	531	OH2	WAT	W	15	22.532	1.843	-4.021	1.00	32.19	W
ATOM	532	OH2	WAT	W	16	23.814	-0.534	-4.336	1.00	39.56	W
ATOM	533	OH2	WAT	W	17	19.996	1.598	-5.292	1.00	33.28	W
ATOM	534	OH2	WAT	W	18	25.262	-3.040	-8.386	1.00	28.37	W
ATOM	535	OH2	WAT	W	19	22.556	0.000	0.001	1.00	30.95	W
ATOM	536	OH2	WAT	W	20	24.369	-1.421	-1.823	1.00	29.32	W
ATOM	537	OH2	WAT	W	21	29.134	-0.583	-6.291	1.00	46.18	W
ATOM	538	OH2	WAT	W	22	27.394	2.286	-5.533	1.00	43.67	W
ATOM	539	OH2	WAT	W	23	26.774	0.049	-4.387	1.00	45.47	W
ATOM	540	OH2	WAT	W	24	30.008	5.236	1.507	1.00	52.80	W
ATOM	541	OH2	WAT	W	25	27.776	4.560	0.356	1.00	42.94	W
ATOM	542	OH2	WAT	W	26	32.018	6.237	0.261	1.00	53.15	W
ATOM	543	OH2	WAT	W	28	18.650	4.426	-0.423	1.00	34.71	W
ATOM	544	OH2	WAT	W	29	18.919	1.842	-1.284	1.00	42.23	W
ATOM	545	OH2	WAT	W	30	11.826	6.239	7.700	1.00	59.49	W
ATOM	546	OH2	WAT	W	31	13.683	5.469	2.919	1.00	52.76	W
ATOM	547	OH2	WAT	W	32	16.956	4.594	1.380	1.00	47.84	W
ATOM	548	OH2	WAT	W	33	17.260	2.099	7.679	1.00	46.32	W
ATOM	549	OH2	WAT	W	34	17.636	1.737	-4.073	1.00	51.94	W
ATOM	550	OH2	WAT	W	35	16.221	5.835	9.764	1.00	30.19	W
ATOM	551	OH2	WAT	W	36	26.030	8.926	8.979	1.00	51.32	W
ATOM	552	OH2	WAT	W	37	13.758	2.898	9.624	1.00	52.05	W
ATOM	553	OH2	WAT	W	38	14.899	5.914	11.925	1.00	35.86	W
ATOM	554	OH2	WAT	W	39	19.841	0.030	14.724	1.00	45.90	W
ATOM	555	OH2	WAT	W	40	13.772	2.335	12.179	1.00	50.60	W
ATOM	556	OH2	WAT	W	41	13.367	0.805	6.229	1.00	51.80	W
ATOM	557	OH2	WAT	W	42	15.587	3.501	15.845	1.00	30.05	W
ATOM	558	OH2	WAT	W	43	14.280	4.098	13.819	1.00	48.74	W
ATOM	559	OH2	WAT	W	44	14.273	3.983	18.042	1.00	32.62	W
ATOM	560	OH2	WAT	W	45	14.275	2.720	20.720	1.00	40.19	W
ATOM	561	OH2	WAT	W	46	21.969	2.228	18.885	1.00	22.32	W
ATOM	562	OH2	WAT	W	47	21.588	1.778	21.594	1.00	28.43	W
ATOM	563	OH2	WAT	W	48	11.908	3.300	22.023	1.00	50.50	W
ATOM	564	OH2	WAT	W	49	13.679	0.626	18.643	1.00	46.64	W
ATOM	565	OH2	WAT	W	50	16.369	2.196	22.597	1.00	30.08	W
ATOM	566	OH2	WAT	W	51	12.828	6.527	18.634	1.00	37.29	W
ATOM	567	OH2	WAT	W	52	24.603	2.631	19.581	1.00	25.55	W
ATOM	568	OH2	WAT	W	53	11.867	0.791	23.131	1.00	58.27	W
ATOM	569	OH2	WAT	W	54	24.646	5.366	17.812	1.00	50.24	W
ATOM	570	OH2	WAT	W	55	20.954	0.091	17.131	1.00	49.14	W
ATOM	571	OH2	WAT	W	56	19.747	-0.562	21.394	1.00	36.92	W
ATOM	572	OH2	WAT	W	57	14.819	8.442	19.922	1.00	33.61	W
ATOM	573	OH2	WAT	W	58	10.854	5.349	19.724	1.00	45.89	W
ATOM	574	OH2	WAT	W	59	10.710	9.378	19.376	1.00	37.52	W
ATOM	575	OH2	WAT	W	60	10.497	10.303	21.845	1.00	34.96	W

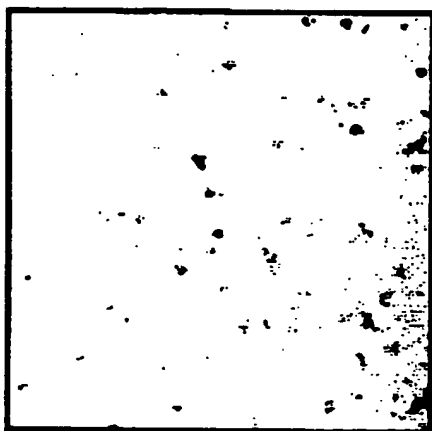
Figure 7L

ATOM	576	OH2	WAT	W	61	12.866	5.691	26.354	1.00	28.86	W
ATOM	577	OH2	WAT	W	62	10.758	7.878	25.495	1.00	42.32	W
ATOM	578	OH2	WAT	W	63	11.782	6.555	28.773	1.00	29.65	W
ATOM	579	OH2	WAT	W	64	10.296	8.472	27.988	1.00	37.31	W
ATOM	580	OH2	WAT	W	65	13.316	2.342	26.849	1.00	43.22	W
ATOM	581	OH2	WAT	W	66	29.863	-1.693	28.654	1.00	38.41	W
ATOM	582	OH2	WAT	W	67	16.468	-1.186	26.444	1.00	32.71	W
ATOM	583	OH2	WAT	W	68	20.934	12.065	25.212	1.00	18.68	W
ATOM	584	OH2	WAT	W	69	7.101	5.989	26.485	1.00	48.02	W
ATOM	585	OH2	WAT	W	70	7.226	10.744	27.574	1.00	33.30	W
ATOM	586	OH2	WAT	W	71	16.382	-1.374	34.997	1.00	34.36	W
ATOM	587	OH2	WAT	W	72	17.474	-0.717	38.167	1.00	28.82	W
ATOM	588	OH2	WAT	W	73	17.984	-2.951	33.186	1.00	27.39	W
ATOM	589	OH2	WAT	W	74	16.999	1.929	37.830	1.00	37.09	W
ATOM	590	OH2	WAT	W	75	20.595	3.071	39.121	1.00	19.51	W
ATOM	591	OH2	WAT	W	76	14.326	5.004	39.584	1.00	20.31	W
ATOM	592	OH2	WAT	W	77	11.973	4.544	38.034	1.00	32.93	W
ATOM	593	OH2	WAT	W	78	18.317	4.417	39.397	1.00	44.00	W
ATOM	594	OH2	WAT	W	79	10.983	-2.804	30.948	1.00	52.39	W
ATOM	595	OH2	WAT	W	80	11.064	0.945	32.640	1.00	30.78	W
ATOM	596	OH2	WAT	W	81	12.861	0.902	39.566	1.00	51.74	W
ATOM	597	OH2	WAT	W	82	14.353	-1.379	39.210	1.00	48.06	W
ATOM	598	OH2	WAT	W	83	13.014	-3.417	36.263	1.00	46.54	W
ATOM	599	OH2	WAT	W	84	11.101	-2.319	39.669	1.00	61.24	W
ATOM	600	OH2	WAT	W	85	20.879	-3.825	31.838	1.00	26.25	W
ATOM	601	OH2	WAT	W	86	24.470	-4.753	28.192	1.00	36.86	W
ATOM	602	OH2	WAT	W	87	22.117	-5.700	29.831	1.00	38.03	W
ATOM	603	OH2	WAT	W	88	19.685	0.721	41.041	1.00	28.21	W
ATOM	604	OH2	WAT	W	89	20.274	5.127	40.337	1.00	32.29	W
ATOM	605	OH2	WAT	W	90	10.072	4.538	29.943	1.00	33.10	W
ATOM	606	OH2	WAT	W	91	10.573	4.216	33.496	1.00	33.22	W
ATOM	607	OH2	WAT	W	92	10.336	5.922	36.364	1.00	48.48	W
ATOM	608	OH2	WAT	W	93	9.113	5.209	40.332	1.00	51.71	W
ATOM	609	OH2	WAT	W	94	9.980	8.713	42.573	1.00	24.98	W
ATOM	610	OH2	WAT	W	95	17.708	6.542	-1.798	1.00	36.93	W
ATOM	611	OH2	WAT	W	96	10.278	11.397	38.730	1.00	17.13	W
ATOM	612	OH2	WAT	W	97	11.290	10.478	36.184	1.00	15.62	W
ATOM	613	OH2	WAT	W	98	8.444	12.988	37.395	1.00	17.25	W
ATOM	614	OH2	WAT	W	99	8.735	9.911	40.361	1.00	25.18	W
ATOM	615	OH2	WAT	W	100	6.665	11.917	35.865	1.00	28.95	W
ATOM	616	OH2	WAT	W	101	8.907	9.736	35.113	1.00	28.77	W
ATOM	617	OH2	WAT	W	102	10.416	5.919	42.300	1.00	32.80	W
ATOM	618	OH2	WAT	W	103	8.278	3.600	38.536	1.00	54.85	W
ATOM	619	OH2	WAT	W	104	14.183	7.249	45.734	1.00	23.53	W
ATOM	620	OH2	WAT	W	105	11.426	7.965	46.547	1.00	34.68	W
ATOM	621	OH2	WAT	W	106	16.907	2.218	41.970	1.00	39.50	W
ATOM	622	OH2	WAT	W	107	16.479	14.336	46.761	1.00	23.72	W
ATOM	623	OH2	WAT	W	108	8.319	12.931	45.022	1.00	22.11	W
ATOM	624	OH2	WAT	W	109	7.189	12.423	42.385	1.00	39.34	W
ATOM	625	OH2	WAT	W	110	8.599	9.769	44.603	1.00	40.15	W
ATOM	626	OH2	WAT	W	111	26.891	-1.858	33.829	1.00	23.69	W
ATOM	627	OH2	WAT	W	112	28.775	-3.310	32.521	1.00	38.13	W
ATOM	628	OH2	WAT	W	113	31.335	0.587	33.068	1.00	34.37	W
ATOM	629	OH2	WAT	W	114	30.921	-0.919	36.513	1.00	44.24	W
ATOM	630	OH2	WAT	W	115	30.098	2.733	29.619	1.00	39.50	W
ATOM	631	OH2	WAT	W	116	33.465	2.665	34.521	1.00	52.27	W
ATOM	632	OH2	WAT	W	117	25.612	14.159	-18.301	1.00	56.10	W
ATOM	633	OH2	WAT	W	118	33.904	2.165	-15.960	1.00	57.70	W

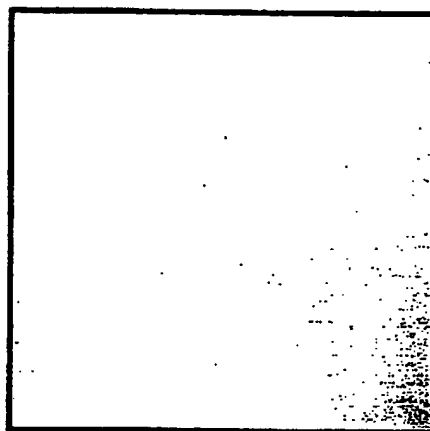
Figure 7M

ATOM	634	OH2	WAT	W	119	33.766	4.315	-14.106	1.00	57.44	W
ATOM	635	OH2	WAT	W	120	26.831	7.497	7.075	1.00	40.38	W
ATOM	636	OH2	WAT	W	121	26.562	8.206	4.240	1.00	32.00	W
ATOM	637	OH2	WAT	W	122	29.081	7.039	3.251	1.00	46.30	W
ATOM	638	OH2	WAT	W	123	22.080	-0.975	10.516	1.00	39.31	W
ATOM	639	OH2	WAT	W	124	28.185	3.991	13.044	1.00	45.28	W
ATOM	640	OH2	WAT	W	125	29.400	7.324	10.996	1.00	52.21	W
ATOM	641	OH2	WAT	W	126	12.966	3.595	24.673	1.00	59.42	W
ATOM	642	OH2	WAT	W	127	8.932	7.961	36.476	1.00	45.85	W
ATOM	643	OH2	WAT	W	128	12.712	5.206	41.719	1.00	38.55	W
ATOM	644	OH2	WAT	W	129	9.431	10.564	47.230	1.00	35.27	W
ATOM	645	OH2	WAT	W	130	6.643	9.576	45.596	1.00	44.00	W
ATOM	646	OH2	WAT	W	131	21.501	13.657	45.856	1.00	43.49	W
ATOM	647	OH2	WAT	W	132	19.368	14.112	46.567	1.00	41.15	W
ATOM	648	OH2	WAT	W	133	20.913	12.058	48.230	1.00	36.86	W
ATOM	649	OH2	WAT	W	134	13.556	4.967	44.137	1.00	49.55	W
ATOM	650	OH2	WAT	W	135	17.568	0.000	0.010	1.00	54.94	W
ATOM	651	OH2	WAT	W	136	17.847	-0.139	11.093	1.00	42.03	W
ATOM	652	OH2	WAT	W	137	25.734	4.074	15.641	1.00	35.36	W
ATOM	653	OH2	WAT	W	138	8.107	7.930	38.831	1.00	37.47	W
ATOM	654	OH2	WAT	W	139	10.614	4.603	44.378	1.00	61.10	W
ATOM	655	OH2	WAT	W	140	14.180	-9.552	32.610	1.00	37.66	W
ATOM	656	OH2	WAT	W	141	26.549	-4.072	22.858	1.00	48.05	W
ATOM	657	OH2	WAT	W	142	21.688	-2.141	22.847	1.00	36.75	W
ATOM	658	OH2	WAT	W	143	15.457	1.462	27.799	1.00	38.11	W
ATOM	659	OH2	WAT	W	144	18.956	16.356	45.521	1.00	36.93	W
ATOM	660	OH2	WAT	W	145	15.655	2.938	40.183	1.00	40.77	W
ATOM	661	OH2	WAT	W	146	15.688	-1.613	19.777	1.00	47.04	W
ATOM	662	OH2	WAT	W	147	26.880	-5.627	28.327	1.00	44.89	W
ATOM	663	OH2	WAT	W	148	28.682	-5.605	33.707	1.00	43.34	W
ATOM	664	OH2	WAT	W	149	28.220	11.179	-23.836	1.00	53.67	W
ATOM	665	OH2	WAT	W	150	27.905	3.222	-7.774	1.00	44.54	W
ATOM	666	OH2	WAT	W	151	15.403	-11.541	32.995	1.00	47.59	W
TER											
END											

Figure 7N

**Inhibition of HIV-1 Membrane Fusion by a D-Peptide****Figure 8A**

Syncytia Assay with no D-peptide

**Figure 8B**

Syncytia Assay with [100 μM] peptide



**NMR Characterization of Aromatic Residues in  
IQN17/D-Peptide Complexes**

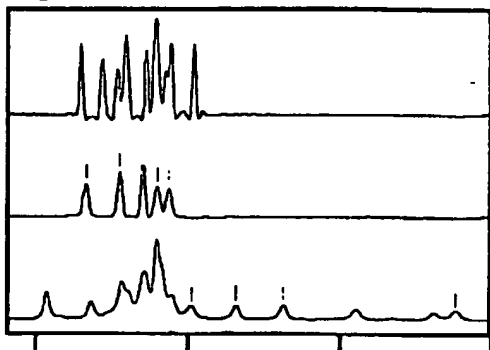
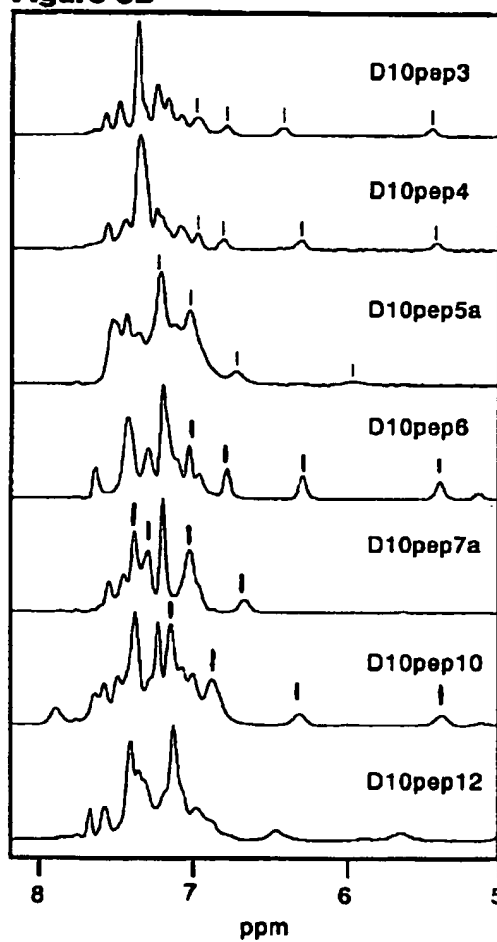
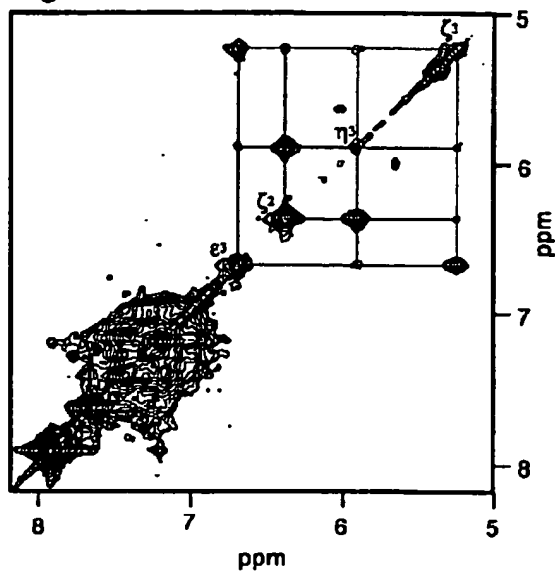
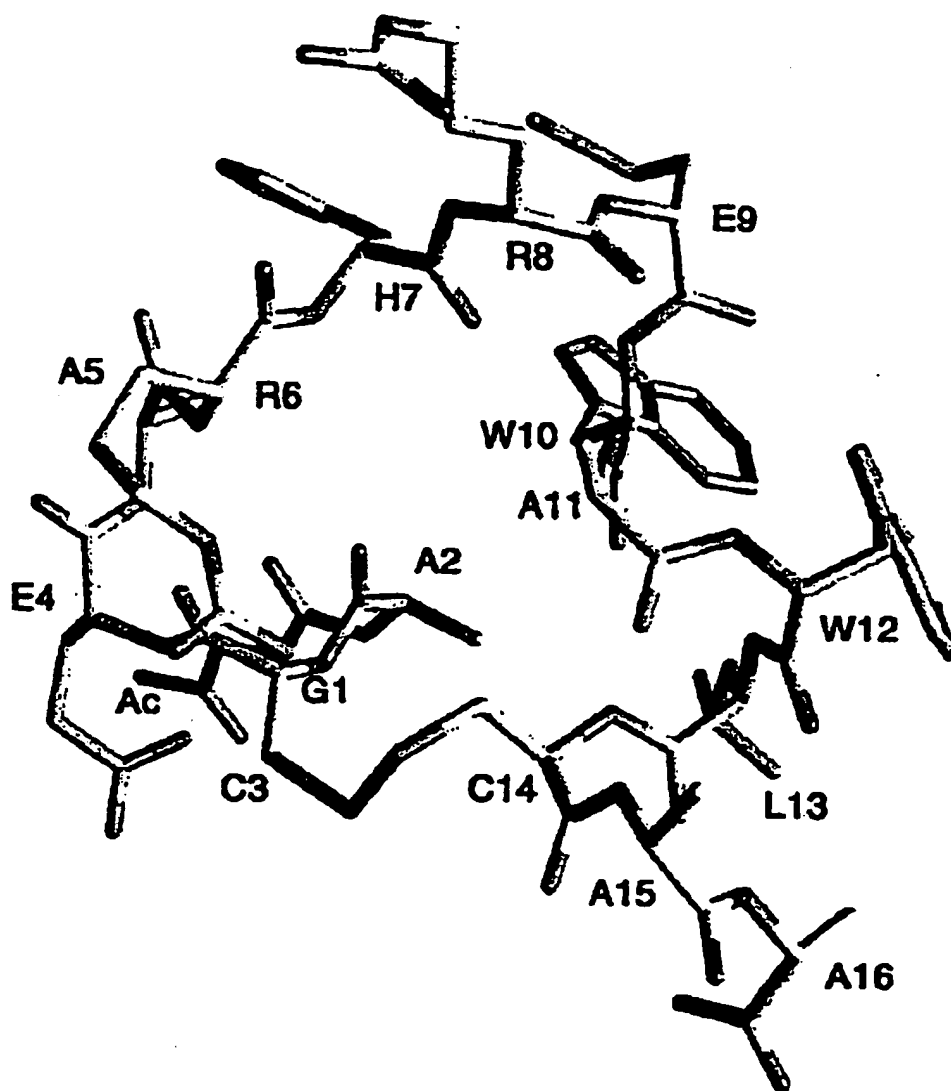
**Figure 9A****Figure 9B****Figure 9C**

Figure 10: Conformation of D10pep1 in complex with IQN17



CRYST1	57.935	121.959	73.669	90.00	90.00	90.00	C2221	1	
ORIGX1	1.000000	0.000000	0.000000			0.000000			
ORIGX2	0.000000	1.000000	0.000000			0.000000			
ORIGX3	0.000000	0.000000	1.000000			0.000000			
SCALE1	0.017261	0.000000	0.000000			0.000000			
SCALE2	0.000000	0.008199	0.000000			0.000000			
SCALE3	0.000000	0.000000	0.013574			0.000000			
ATOM	1	CA	ACE	A	0	25.795	17.140	37.286	1.00 61.88 A
ATOM	2	C	ACE	A	0	25.799	18.376	36.435	1.00 62.00 A
ATOM	3	O	ACE	A	0	25.500	19.475	36.921	1.00 62.10 A
ATOM	4	N	ARG	A	1	26.134	18.217	35.157	1.00 60.34 A
ATOM	5	CA	ARG	A	1	26.203	19.328	34.217	1.00 60.56 A
ATOM	6	CB	ARG	A	1	27.212	18.993	33.110	1.00 61.87 A
ATOM	7	CG	ARG	A	1	27.630	20.135	32.212	1.00 60.78 A
ATOM	8	CD	ARG	A	1	28.500	19.587	31.097	1.00 64.25 A
ATOM	9	NE	ARG	A	1	29.018	20.628	30.217	1.00 65.07 A
ATOM	10	CZ	ARG	A	1	29.706	20.377	29.109	1.00 63.90 A
ATOM	11	NH1	ARG	A	1	29.951	19.124	28.766	1.00 64.20 A
ATOM	12	NH2	ARG	A	1	30.157	21.367	28.351	1.00 63.51 A
ATOM	13	C	ARG	A	1	24.823	19.573	33.595	1.00 59.45 A
ATOM	14	O	ARG	A	1	24.453	20.714	33.294	1.00 57.69 A
ATOM	15	N	MET	A	2	24.065	18.494	33.425	1.00 57.60 A
ATOM	16	CA	MET	A	2	22.736	18.573	32.836	1.00 59.85 A
ATOM	17	CB	MET	A	2	22.273	17.198	32.397	1.00 59.85 A
ATOM	18	CG	MET	A	2	21.204	17.251	31.342	1.00 63.56 A
ATOM	19	SD	MET	A	2	20.044	15.905	31.454	1.00 67.77 A
ATOM	20	CE	MET	A	2	19.089	16.438	32.857	1.00 66.61 A
ATOM	21	C	MET	A	2	21.723	19.130	33.834	1.00 61.33 A
ATOM	22	O	MET	A	2	20.543	19.276	33.521	1.00 59.97 A
ATOM	23	N	LYS	A	3	22.200	19.417	35.041	1.00 62.71 A
ATOM	24	CA	LYS	A	3	21.373	19.961	36.107	1.00 63.07 A
ATOM	25	CB	LYS	A	3	21.817	19.361	37.449	1.00 64.25 A
ATOM	26	CG	LYS	A	3	20.982	19.721	38.687	1.00 64.89 A
ATOM	27	CD	LYS	A	3	21.195	21.159	39.160	1.00 64.67 A
ATOM	28	CE	LYS	A	3	20.543	21.405	40.525	1.00 64.66 A
ATOM	29	NZ	LYS	A	3	19.077	21.123	40.548	1.00 63.04 A
ATOM	30	C	LYS	A	3	21.599	21.467	36.062	1.00 64.55 A
ATOM	31	O	LYS	A	3	20.639	22.245	36.032	1.00 64.65 A
ATOM	32	N	GLN	A	4	22.869	21.873	36.036	1.00 64.34 A
ATOM	33	CA	GLN	A	4	23.232	23.289	35.952	1.00 65.46 A
ATOM	34	CB	GLN	A	4	24.746	23.447	35.780	1.00 67.71 A
ATOM	35	CG	GLN	A	4	25.552	22.954	36.963	1.00 71.16 A
ATOM	36	CD	GLN	A	4	25.297	23.771	38.212	1.00 75.18 A
ATOM	37	OE1	GLN	A	4	25.618	24.962	38.269	1.00 77.70 A
ATOM	38	NE2	GLN	A	4	24.706	23.135	39.225	1.00 76.77 A
ATOM	39	C	GLN	A	4	22.508	23.928	34.758	1.00 64.11 A
ATOM	40	O	GLN	A	4	22.191	25.128	34.776	1.00 62.08 A
ATOM	41	N	ILE	A	5	22.260	23.120	33.726	1.00 59.80 A
ATOM	42	CA	ILE	A	5	21.540	23.587	32.552	1.00 58.22 A
ATOM	43	CB	ILE	A	5	21.567	22.558	31.398	1.00 56.85 A
ATOM	44	CG2	ILE	A	5	20.438	22.851	30.416	1.00 53.92 A
ATOM	45	CG1	ILE	A	5	22.942	22.562	30.719	1.00 56.47 A
ATOM	46	CD1	ILE	A	5	23.079	21.524	29.614	1.00 59.50 A
ATOM	47	C	ILE	A	5	20.083	23.828	32.929	1.00 58.98 A
ATOM	48	O	ILE	A	5	19.575	24.928	32.729	1.00 58.48 A
ATOM	49	N	GLU	A	6	19.424	22.796	33.472	1.00 59.29 A
ATOM	50	CA	GLU	A	6	18.013	22.883	33.377	1.00 56.51 A
ATOM	51	CB	GLU	A	6	17.528	21.537	34.448	1.00 55.59 A

Figure 11A

ATOM	52	CG	GLU	A	6	17.636	20.359	33.480	1.00	56.46	A
ATOM	53	CD	GLU	A	6	17.293	19.009	34.119	1.00	56.33	A
ATOM	54	OE1	GLU	A	6	17.702	18.790	35.278	1.00	53.43	A
ATOM	55	OE2	GLU	A	6	16.644	18.157	33.458	1.00	55.03	A
ATOM	56	C	GLU	A	6	17.873	23.977	34.926	1.00	54.87	A
ATOM	57	O	GLU	A	6	16.793	24.509	35.137	1.00	52.82	A
ATOM	58	N	ASP	A	7	18.986	24.300	35.572	1.00	55.62	A
ATOM	59	CA	ASP	A	7	19.039	25.336	36.597	1.00	56.65	A
ATOM	60	CB	ASP	A	7	20.291	25.162	37.451	1.00	57.46	A
ATOM	61	CG	ASP	A	7	20.010	24.471	38.762	1.00	57.37	A
ATOM	62	OD1	ASP	A	7	19.180	23.534	38.775	1.00	53.78	A
ATOM	63	OD2	ASP	A	7	20.637	24.862	39.771	1.00	57.66	A
ATOM	64	C	ASP	A	7	19.034	26.745	36.041	1.00	56.99	A
ATOM	65	O	ASP	A	7	18.516	27.662	36.678	1.00	55.43	A
ATOM	66	N	LYS	A	8	19.632	26.945	34.873	1.00	58.30	A
ATOM	67	CA	LYS	A	8	19.642	28.290	34.312	1.00	59.87	A
ATOM	68	CB	LYS	A	8	20.971	28.599	33.612	1.00	62.61	A
ATOM	69	CG	LYS	A	8	22.203	28.372	34.487	1.00	66.85	A
ATOM	70	CD	LYS	A	8	23.232	29.498	34.357	1.00	70.21	A
ATOM	71	CE	LYS	A	8	22.915	30.676	35.293	1.00	72.00	A
ATOM	72	NZ	LYS	A	8	21.583	31.323	35.091	1.00	72.05	A
ATOM	73	C	LYS	A	8	18.467	28.481	33.354	1.00	58.08	A
ATOM	74	O	LYS	A	8	18.145	29.609	32.969	1.00	56.44	A
ATOM	75	N	ILE	A	9	17.835	27.376	32.967	1.00	55.29	A
ATOM	76	CA	ILE	A	9	16.668	27.436	32.099	1.00	56.69	A
ATOM	77	CB	ILE	A	9	16.325	26.052	31.486	1.00	54.89	A
ATOM	78	CG2	ILE	A	9	14.892	26.067	30.915	1.00	54.20	A
ATOM	79	CG1	ILE	A	9	17.373	25.676	30.423	1.00	55.96	A
ATOM	80	CD1	ILE	A	9	17.131	24.339	29.717	1.00	54.22	A
ATOM	81	C	ILE	A	9	15.526	27.876	33.018	1.00	57.98	A
ATOM	82	O	ILE	A	9	14.603	28.572	32.616	1.00	55.85	A
ATOM	83	N	GLU	A	10	15.626	27.458	34.271	1.00	59.96	A
ATOM	84	CA	GLU	A	10	14.641	27.788	35.283	1.00	61.12	A
ATOM	85	CB	GLU	A	10	14.850	26.901	36.510	1.00	63.01	A
ATOM	86	CG	GLU	A	10	13.846	27.117	37.618	1.00	66.89	A
ATOM	87	CD	GLU	A	10	14.387	26.672	38.955	1.00	68.37	A
ATOM	88	OE1	GLU	A	10	14.844	25.510	39.054	1.00	67.70	A
ATOM	89	OE2	GLU	A	10	14.355	27.487	39.903	1.00	68.42	A
ATOM	90	C	GLU	A	10	14.872	29.243	35.664	1.00	59.41	A
ATOM	91	O	GLU	A	10	13.947	29.958	36.037	1.00	59.95	A
ATOM	92	N	GLU	A	11	16.127	29.663	35.565	1.00	57.16	A
ATOM	93	CA	GLU	A	11	16.524	31.024	35.893	1.00	55.88	A
ATOM	94	CB	GLU	A	11	18.042	31.095	36.019	1.00	58.17	A
ATOM	95	CG	GLU	A	11	18.569	32.375	36.627	1.00	62.73	A
ATOM	96	CD	GLU	A	11	18.459	32.382	38.139	1.00	67.75	A
ATOM	97	OE1	GLU	A	11	19.101	31.512	38.782	1.00	67.91	A
ATOM	98	OE2	GLU	A	11	17.736	33.249	38.681	1.00	68.84	A
ATOM	99	C	GLU	A	11	16.056	31.976	34.789	1.00	54.76	A
ATOM	100	O	GLU	A	11	15.805	33.160	35.030	1.00	54.78	A
ATOM	101	N	ILE	A	12	15.945	31.443	33.575	1.00	52.61	A
ATOM	102	CA	ILE	A	12	15.510	32.210	32.414	1.00	50.09	A
ATOM	103	CB	ILE	A	12	16.002	31.548	31.096	1.00	50.23	A
ATOM	104	CG2	ILE	A	12	15.201	32.073	29.905	1.00	48.54	A
ATOM	105	CG1	ILE	A	12	17.508	31.773	30.930	1.00	50.30	A
ATOM	106	CD1	ILE	A	12	18.114	31.062	29.724	1.00	53.10	A
ATOM	107	C	ILE	A	12	13.988	32.324	32.362	1.00	49.83	A
ATOM	108	O	ILE	A	12	13.447	33.376	32.017	1.00	47.70	A
ATOM	109	N	GLU	A	13	13.306	31.232	32.698	1.00	48.57	A
ATOM	110	CA	GLU	A	13	11.849	31.219	32.677	1.00	48.22	A
ATOM	111	CB	GLU	A	13	11.320	29.810	32.954	1.00	45.44	A

Figure 11B

ATOM	112	CG	GLU	A	13	11.673	28.794	31.895	1.00	46.65	A
ATOM	113	CD	GLU	A	13	11.419	27.372	32.358	1.00	49.90	A
ATOM	114	OE1	GLU	A	13	12.051	26.968	33.366	1.00	49.96	A
ATOM	115	OE2	GLU	A	13	10.599	26.665	31.720	1.00	50.18	A
ATOM	116	C	GLU	A	13	11.357	32.163	33.749	1.00	47.83	A
ATOM	117	O	GLU	A	13	10.279	32.731	33.638	1.00	48.72	A
ATOM	118	N	SER	A	14	12.168	32.313	34.786	1.00	48.67	A
ATOM	119	CA	SER	A	14	11.862	33.187	35.907	1.00	49.89	A
ATOM	120	CB	SER	A	14	12.906	32.985	37.014	1.00	49.05	A
ATOM	121	OG	SER	A	14	12.634	33.773	38.160	1.00	49.35	A
ATOM	122	C	SER	A	14	11.885	34.627	35.415	1.00	50.52	A
ATOM	123	O	SER	A	14	10.869	35.313	35.431	1.00	54.15	A
ATOM	124	N	LYS	A	15	13.056	35.067	34.971	1.00	49.27	A
ATOM	125	CA	LYS	A	15	13.248	36.416	34.474	1.00	51.02	A
ATOM	126	CB	LYS	A	15	14.707	36.589	34.042	1.00	54.30	A
ATOM	127	CG	LYS	A	15	15.018	37.931	33.417	1.00	58.79	A
ATOM	128	CD	LYS	A	15	14.843	39.039	34.437	1.00	63.42	A
ATOM	129	CE	LYS	A	15	15.841	38.880	35.576	1.00	65.66	A
ATOM	130	NZ	LYS	A	15	15.722	39.983	36.569	1.00	68.14	A
ATOM	131	C	LYS	A	15	12.313	36.758	33.305	1.00	50.99	A
ATOM	132	O	LYS	A	15	12.022	37.926	33.061	1.00	49.62	A
ATOM	133	N	GLN	A	16	11.848	35.740	32.587	1.00	50.06	A
ATOM	134	CA	GLN	A	16	10.965	35.937	31.444	1.00	49.96	A
ATOM	135	CB	GLN	A	16	10.950	34.684	30.570	1.00	49.89	A
ATOM	136	CG	GLN	A	16	10.133	34.810	29.286	1.00	50.59	A
ATOM	137	CD	GLN	A	16	10.287	33.603	28.369	1.00	54.27	A
ATOM	138	OE1	GLN	A	16	9.799	32.511	28.667	1.00	56.28	A
ATOM	139	NE2	GLN	A	16	10.985	33.796	27.250	1.00	54.69	A
ATOM	140	C	GLN	A	16	9.551	36.256	31.899	1.00	50.61	A
ATOM	141	O	GLN	A	16	8.788	36.931	31.195	1.00	48.56	A
ATOM	142	N	LYS	A	17	9.198	35.736	33.067	1.00	49.38	A
ATOM	143	CA	LYS	A	17	7.883	35.973	33.623	1.00	49.73	A
ATOM	144	CB	LYS	A	17	7.582	34.982	34.750	1.00	52.97	A
ATOM	145	CG	LYS	A	17	6.250	35.226	35.448	1.00	56.86	A
ATOM	146	CD	LYS	A	17	6.066	34.276	36.618	1.00	59.31	A
ATOM	147	CE	LYS	A	17	4.763	34.552	37.354	1.00	59.95	A
ATOM	148	NZ	LYS	A	17	4.592	33.621	38.506	1.00	62.05	A
ATOM	149	C	LYS	A	17	7.927	37.390	34.163	1.00	48.25	A
ATOM	150	O	LYS	A	17	6.977	38.144	34.008	1.00	47.73	A
ATOM	151	N	LYS	A	18	9.043	37.750	34.791	1.00	45.58	A
ATOM	152	CA	LYS	A	18	9.190	39.101	35.309	1.00	45.26	A
ATOM	153	CB	LYS	A	18	10.523	39.270	36.047	1.00	47.34	A
ATOM	154	CG	LYS	A	18	10.627	38.493	37.362	1.00	50.10	A
ATOM	155	CD	LYS	A	18	11.831	38.976	38.168	1.00	52.93	A
ATOM	156	CE	LYS	A	18	11.869	38.358	39.550	1.00	55.07	A
ATOM	157	NZ	LYS	A	18	12.933	38.968	40.398	1.00	59.20	A
ATOM	158	C	LYS	A	18	9.107	40.110	34.171	1.00	41.59	A
ATOM	159	O	LYS	A	18	8.585	41.206	34.349	1.00	42.70	A
ATOM	160	N	ILE	A	19	9.633	39.740	33.008	1.00	40.25	A
ATOM	161	CA	ILE	A	19	9.605	40.595	31.831	1.00	39.53	A
ATOM	162	CB	ILE	A	19	10.494	40.015	30.710	1.00	42.08	A
ATOM	163	CG2	ILE	A	19	10.133	40.631	29.369	1.00	41.71	A
ATOM	164	CG1	ILE	A	19	11.969	40.214	31.074	1.00	42.52	A
ATOM	165	CD1	ILE	A	19	12.939	39.656	30.039	1.00	43.29	A
ATOM	166	C	ILE	A	19	8.172	40.725	31.325	1.00	39.27	A
ATOM	167	O	ILE	A	19	7.751	41.790	30.899	1.00	37.81	A
ATOM	168	N	GLU	A	20	7.421	39.637	31.372	1.00	39.00	A
ATOM	169	CA	GLU	A	20	6.036	39.692	30.930	1.00	40.27	A
ATOM	170	CB	GLU	A	20	5.437	38.280	30.834	1.00	43.21	A
ATOM	171	CG	GLU	A	20	5.898	37.474	29.606	1.00	48.10	A

Figure 11C

ATOM	172	CD	GLU	A	20	5.446	36.019	29.659	1.00	50.57	A
ATOM	173	OE1	GLU	A	20	5.832	35.316	30.617	1.00	52.42	A
ATOM	174	OE2	GLU	A	20	4.708	35.575	28.752	1.00	52.16	A
ATOM	175	C	GLU	A	20	5.195	40.546	31.873	1.00	40.09	A
ATOM	176	O	GLU	A	20	4.148	41.056	31.480	1.00	40.96	A
ATOM	177	N	ASN	A	21	5.637	40.694	33.119	1.00	38.83	A
ATOM	178	CA	ASN	A	21	4.880	41.498	34.071	1.00	40.69	A
ATOM	179	CB	ASN	A	21	5.216	41.107	35.507	1.00	39.42	A
ATOM	180	CG	ASN	A	21	4.618	39.768	35.892	1.00	41.35	A
ATOM	181	OD1	ASN	A	21	3.905	39.151	35.102	1.00	38.98	A
ATOM	182	ND2	ASN	A	21	4.902	39.312	37.107	1.00	40.82	A
ATOM	183	C	ASN	A	21	5.163	42.958	33.846	1.00	42.25	A
ATOM	184	O	ASN	A	21	4.261	43.801	33.872	1.00	42.61	A
ATOM	185	N	GLU	A	22	6.432	43.244	33.602	1.00	41.94	A
ATOM	186	CA	GLU	A	22	6.893	44.589	33.343	1.00	41.44	A
ATOM	187	CB	GLU	A	22	8.403	44.563	33.127	1.00	43.01	A
ATOM	188	CG	GLU	A	22	9.126	45.861	33.421	1.00	49.75	A
ATOM	189	CD	GLU	A	22	9.769	45.872	34.802	1.00	52.80	A
ATOM	190	OE1	GLU	A	22	10.611	44.988	35.077	1.00	53.66	A
ATOM	191	OE2	GLU	A	22	9.447	46.764	35.608	1.00	57.41	A
ATOM	192	C	GLU	A	22	6.188	45.082	32.068	1.00	41.34	A
ATOM	193	O	GLU	A	22	5.851	46.263	31.954	1.00	43.52	A
ATOM	194	N	ILE	A	23	5.964	44.175	31.116	1.00	37.55	A
ATOM	195	CA	ILE	A	23	5.295	44.530	29.863	1.00	35.10	A
ATOM	196	CB	ILE	A	23	5.418	43.408	28.800	1.00	36.19	A
ATOM	197	CG2	ILE	A	23	4.520	43.719	27.592	1.00	35.94	A
ATOM	198	CG1	ILE	A	23	6.876	43.288	28.340	1.00	39.18	A
ATOM	199	CD1	ILE	A	23	7.122	42.193	27.324	1.00	40.80	A
ATOM	200	C	ILE	A	23	3.816	44.827	30.093	1.00	33.36	A
ATOM	201	O	ILE	A	23	3.284	45.796	29.568	1.00	28.55	A
ATOM	202	N	ALA	A	24	3.167	43.981	30.881	1.00	30.41	A
ATOM	203	CA	ALA	A	24	1.760	44.147	31.179	1.00	30.11	A
ATOM	204	CB	ALA	A	24	1.276	42.994	32.043	1.00	27.29	A
ATOM	205	C	ALA	A	24	1.531	45.479	31.893	1.00	31.41	A
ATOM	206	O	ALA	A	24	0.562	46.183	31.608	1.00	31.49	A
ATOM	207	N	ARG	A	25	2.428	45.825	32.816	1.00	30.94	A
ATOM	208	CA	ARG	A	25	2.297	47.070	33.547	1.00	30.44	A
ATOM	209	CB	ARG	A	25	3.197	47.066	34.798	1.00	32.01	A
ATOM	210	CG	ARG	A	25	2.727	46.101	35.894	1.00	34.49	A
ATOM	211	CD	ARG	A	25	3.471	46.326	37.218	1.00	39.65	A
ATOM	212	NE	ARG	A	25	4.873	45.907	37.177	1.00	40.74	A
ATOM	213	CZ	ARG	A	25	5.308	44.687	37.496	1.00	43.06	A
ATOM	214	NH1	ARG	A	25	4.453	43.749	37.885	1.00	39.85	A
ATOM	215	NH2	ARG	A	25	6.606	44.399	37.399	1.00	40.30	A
ATOM	216	C	ARG	A	25	2.590	48.270	32.651	1.00	28.86	A
ATOM	217	O	ARG	A	25	1.907	49.296	32.728	1.00	29.35	A
ATOM	218	N	ILE	A	26	3.587	48.147	31.790	1.00	26.96	A
ATOM	219	CA	ILE	A	26	3.917	49.226	30.875	1.00	29.07	A
ATOM	220	CB	ILE	A	26	5.132	48.832	29.990	1.00	28.43	A
ATOM	221	CG2	ILE	A	26	5.239	49.760	28.799	1.00	25.38	A
ATOM	222	CG1	ILE	A	26	6.414	48.835	30.839	1.00	28.70	A
ATOM	223	CD1	ILE	A	26	7.646	48.257	30.132	1.00	27.77	A
ATOM	224	C	ILE	A	26	2.719	49.571	29.968	1.00	30.92	A
ATOM	225	O	ILE	A	26	2.435	50.746	29.690	1.00	32.33	A
ATOM	226	N	LYS	A	27	2.019	48.540	29.512	1.00	30.36	A
ATOM	227	CA	LYS	A	27	0.887	48.730	28.627	1.00	30.40	A
ATOM	228	CB	LYS	A	27	0.449	47.388	28.045	1.00	33.83	A
ATOM	229	CG	LYS	A	27	1.520	46.729	27.185	1.00	39.64	A
ATOM	230	CD	LYS	A	27	1.167	45.294	26.831	1.00	44.41	A
ATOM	231	CE	LYS	A	27	-0.086	45.204	26.003	1.00	46.84	A

Figure 11D

ATOM	232	NZ	LYS	A	27	-0.384	43.774	25.698	1.00	53.94	A
ATOM	233	C	LYS	A	27	-0.267	49.402	29.344	1.00	28.67	A
ATOM	234	O	LYS	A	27	-0.919	50.252	28.767	1.00	26.05	A
ATOM	235	N	LYS	A	28	-0.511	49.020	30.593	1.00	27.68	A
ATOM	236	CA	LYS	A	28	-1.597	49.609	31.371	1.00	27.30	A
ATOM	237	CB	LYS	A	28	-1.797	48.845	32.691	1.00	24.82	A
ATOM	238	CG	LYS	A	28	-2.961	49.384	33.573	1.00	27.48	A
ATOM	239	CD	LYS	A	28	-4.263	49.506	32.744	1.00	31.59	A
ATOM	240	CE	LYS	A	28	-5.526	49.699	33.606	1.00	30.02	A
ATOM	241	NZ	LYS	A	28	-5.440	50.820	34.586	1.00	31.11	A
ATOM	242	C	LYS	A	28	-1.284	51.076	31.641	1.00	29.57	A
ATOM	243	O	LYS	A	28	-2.164	51.951	31.566	1.00	28.21	A
ATOM	244	N	LEU	A	29	-0.017	51.359	31.923	1.00	29.36	A
ATOM	245	CA	LEU	A	29	0.385	52.723	32.179	1.00	33.70	A
ATOM	246	CB	LEU	A	29	1.822	52.745	32.692	1.00	35.26	A
ATOM	247	CG	LEU	A	29	2.023	53.727	33.847	1.00	38.04	A
ATOM	248	CD1	LEU	A	29	3.363	53.485	34.506	1.00	39.85	A
ATOM	249	CD2	LEU	A	29	1.891	55.149	33.332	1.00	38.01	A
ATOM	250	C	LEU	A	29	0.243	53.561	30.905	1.00	34.59	A
ATOM	251	O	LEU	A	29	-0.281	54.691	30.927	1.00	37.16	A
ATOM	252	N	LEU	A	30	0.721	53.020	29.792	1.00	34.03	A
ATOM	253	CA	LEU	A	30	0.616	53.724	28.528	1.00	35.56	A
ATOM	254	CB	LEU	A	30	1.230	52.874	27.414	1.00	38.09	A
ATOM	255	CG	LEU	A	30	1.470	53.508	26.050	1.00	40.19	A
ATOM	256	CD1	LEU	A	30	2.270	54.805	26.163	1.00	39.79	A
ATOM	257	CD2	LEU	A	30	2.215	52.484	25.198	1.00	45.44	A
ATOM	258	C	LEU	A	30	-0.882	53.980	28.263	1.00	34.76	A
ATOM	259	O	LEU	A	30	-1.269	55.050	27.794	1.00	33.56	A
ATOM	260	N	GLN	A	31	-1.713	52.996	28.572	1.00	30.55	A
ATOM	261	CA	GLN	A	31	-3.152	53.142	28.401	1.00	31.04	A
ATOM	262	CB	GLN	A	31	-3.865	51.839	28.782	1.00	33.01	A
ATOM	263	CG	GLN	A	31	-5.397	51.924	28.839	1.00	37.09	A
ATOM	264	CD	GLN	A	31	-6.045	50.582	29.159	1.00	45.53	A
ATOM	265	OE1	GLN	A	31	-5.715	49.940	30.159	1.00	52.72	A
ATOM	266	NE2	GLN	A	31	-6.973	50.151	28.310	1.00	46.91	A
ATOM	267	C	GLN	A	31	-3.633	54.303	29.273	1.00	31.34	A
ATOM	268	O	GLN	A	31	-4.419	55.125	28.832	1.00	28.45	A
ATOM	269	N	LEU	A	32	-3.141	54.376	30.509	1.00	30.93	A
ATOM	270	CA	LEU	A	32	-3.523	55.459	31.393	1.00	30.83	A
ATOM	271	CB	LEU	A	32	-2.988	55.237	32.811	1.00	29.49	A
ATOM	272	CG	LEU	A	32	-3.572	54.156	33.732	1.00	31.79	A
ATOM	273	CD1	LEU	A	32	-2.810	54.215	35.075	1.00	33.29	A
ATOM	274	CD2	LEU	A	32	-5.058	54.376	33.972	1.00	25.39	A
ATOM	275	C	LEU	A	32	-3.031	56.797	30.860	1.00	32.26	A
ATOM	276	O	LEU	A	32	-3.707	57.810	31.031	1.00	35.77	A
ATOM	277	N	THR	A	33	-1.872	56.798	30.198	1.00	31.70	A
ATOM	278	CA	THR	A	33	-1.298	58.019	29.640	1.00	33.33	A
ATOM	279	CB	THR	A	33	0.158	57.787	29.156	1.00	35.07	A
ATOM	280	OG1	THR	A	33	0.949	57.272	30.238	1.00	39.00	A
ATOM	281	CG2	THR	A	33	0.776	59.087	28.687	1.00	34.58	A
ATOM	282	C	THR	A	33	-2.120	58.560	28.471	1.00	33.63	A
ATOM	283	O	THR	A	33	-2.237	59.767	28.298	1.00	33.87	A
ATOM	284	N	VAL	A	34	-2.682	57.660	27.670	1.00	35.32	A
ATOM	285	CA	VAL	A	34	-3.507	58.046	26.531	1.00	36.90	A
ATOM	286	CB	VAL	A	34	-3.810	56.832	25.622	1.00	36.47	A
ATOM	287	CG1	VAL	A	34	-4.825	57.200	24.550	1.00	34.36	A
ATOM	288	CG2	VAL	A	34	-2.514	56.354	24.966	1.00	38.97	A
ATOM	289	C	VAL	A	34	-4.809	58.655	27.036	1.00	37.01	A
ATOM	290	O	VAL	A	34	-5.250	59.695	26.540	1.00	35.59	A
ATOM	291	N	TRP	A	35	-5.403	57.992	28.022	1.00	36.34	A

Figure 11E

ATOM	292	CA	TRP	A	35	-6.645	58.429	28.648	1.00	38.95	A
ATOM	293	CB	TRP	A	35	-7.022	57.429	29.742	1.00	44.03	A
ATOM	294	CG	TRP	A	35	-8.302	57.716	30.478	1.00	45.10	A
ATOM	295	CD2	TRP	A	35	-8.445	58.535	31.640	1.00	46.19	A
ATOM	296	CE2	TRP	A	35	-9.820	58.545	31.973	1.00	47.39	A
ATOM	297	CE3	TRP	A	35	-7.556	59.277	32.429	1.00	46.15	A
ATOM	298	CD1	TRP	A	35	-9.549	57.260	30.166	1.00	45.42	A
ATOM	299	NE1	TRP	A	35	-10.468	57.752	31.063	1.00	47.75	A
ATOM	300	CZ2	TRP	A	35	-10.317	59.258	33.067	1.00	48.12	A
ATOM	301	CZ3	TRP	A	35	-8.049	59.991	33.509	1.00	44.34	A
ATOM	302	CH2	TRP	A	35	-9.419	59.968	33.824	1.00	47.03	A
ATOM	303	C	TRP	A	35	-6.408	59.814	29.259	1.00	40.04	A
ATOM	304	O	TRP	A	35	-7.153	60.759	29.013	1.00	39.15	A
ATOM	305	N	GLY	A	36	-5.352	59.934	30.055	1.00	38.98	A
ATOM	306	CA	GLY	A	36	-5.039	61.211	30.658	1.00	38.44	A
ATOM	307	C	GLY	A	36	-5.034	62.327	29.634	1.00	38.41	A
ATOM	308	O	GLY	A	36	-5.626	63.390	29.845	1.00	40.58	A
ATOM	309	N	ILE	A	37	-4.356	62.094	28.517	1.00	39.01	A
ATOM	310	CA	ILE	A	37	-4.279	63.079	27.451	1.00	40.60	A
ATOM	311	CB	ILE	A	37	-3.395	62.584	26.301	1.00	40.20	A
ATOM	312	CG2	ILE	A	37	-3.509	63.517	25.136	1.00	39.97	A
ATOM	313	CG1	ILE	A	37	-1.939	62.477	26.767	1.00	41.25	A
ATOM	314	CD1	ILE	A	37	-1.036	61.777	25.778	1.00	38.31	A
ATOM	315	C	ILE	A	37	-5.662	63.366	26.886	1.00	42.00	A
ATOM	316	O	ILE	A	37	-6.019	64.516	26.654	1.00	42.52	A
ATOM	317	N	LYS	A	38	-6.438	62.317	26.660	1.00	42.56	A
ATOM	318	CA	LYS	A	38	-7.766	62.505	26.112	1.00	45.16	A
ATOM	319	CB	LYS	A	38	-8.459	61.156	25.925	1.00	46.50	A
ATOM	320	CG	LYS	A	38	-9.683	61.235	25.026	1.00	53.52	A
ATOM	321	CD	LYS	A	38	-10.840	62.017	25.651	1.00	55.55	A
ATOM	322	CE	LYS	A	38	-11.812	62.480	24.581	1.00	56.01	A
ATOM	323	NZ	LYS	A	38	-11.165	63.504	23.714	1.00	55.27	A
ATOM	324	C	LYS	A	38	-8.594	63.405	27.025	1.00	46.34	A
ATOM	325	O	LYS	A	38	-9.237	64.343	26.561	1.00	48.52	A
ATOM	326	N	GLN	A	39	-8.554	63.120	28.322	1.00	47.82	A
ATOM	327	CA	GLN	A	39	-9.303	63.877	29.318	1.00	49.21	A
ATOM	328	CB	GLN	A	39	-9.142	63.230	30.691	1.00	52.07	A
ATOM	329	CG	GLN	A	39	-9.431	61.742	30.727	1.00	59.01	A
ATOM	330	CD	GLN	A	39	-10.889	61.409	30.513	1.00	61.01	A
ATOM	331	OE1	GLN	A	39	-11.742	61.800	31.310	1.00	63.56	A
ATOM	332	NE2	GLN	A	39	-11.188	60.677	29.437	1.00	62.00	A
ATOM	333	C	GLN	A	39	-8.840	65.324	29.412	1.00	48.78	A
ATOM	334	O	GLN	A	39	-9.649	66.243	29.431	1.00	48.03	A
ATOM	335	N	LEU	A	40	-7.530	65.522	29.472	1.00	49.67	A
ATOM	336	CA	LEU	A	40	-6.980	66.861	29.590	1.00	50.78	A
ATOM	337	CB	LEU	A	40	-5.479	66.785	29.868	1.00	49.62	A
ATOM	338	CG	LEU	A	40	-4.736	68.118	29.982	1.00	47.99	A
ATOM	339	CD1	LEU	A	40	-5.416	69.030	31.011	1.00	51.32	A
ATOM	340	CD2	LEU	A	40	-3.300	67.852	30.376	1.00	48.82	A
ATOM	341	C	LEU	A	40	-7.227	67.736	28.363	1.00	53.20	A
ATOM	342	O	LEU	A	40	-7.230	68.964	28.457	1.00	53.67	A
ATOM	343	N	GLN	A	41	-7.433	67.104	27.215	1.00	56.61	A
ATOM	344	CA	GLN	A	41	-7.649	67.850	25.994	1.00	60.81	A
ATOM	345	CB	GLN	A	41	-7.295	66.994	24.781	1.00	60.00	A
ATOM	346	CG	GLN	A	41	-7.257	67.753	23.467	1.00	61.60	A
ATOM	347	CD	GLN	A	41	-6.756	66.885	22.330	1.00	61.14	A
ATOM	348	OE1	GLN	A	41	-5.630	66.377	22.367	1.00	56.12	A
ATOM	349	NE2	GLN	A	41	-7.598	66.697	21.316	1.00	60.61	A
ATOM	350	C	GLN	A	41	-9.084	68.344	25.915	1.00	63.54	A
ATOM	351	O	GLN	A	41	-9.388	69.277	25.179	1.00	65.13	A

Figure 11F



ATOM	352	N	ALA	A	42	-9.971	67.722	26.679	1.00	67.16	A
ATOM	353	CA	ALA	A	42	-11.362	68.150	26.693	1.00	70.08	A
ATOM	354	CB	ALA	A	42	-12.252	67.043	27.249	1.00	68.59	A
ATOM	355	C	ALA	A	42	-11.461	69.423	27.556	1.00	72.76	A
ATOM	356	O	ALA	A	42	-12.506	69.748	28.123	1.00	73.45	A
ATOM	357	N	ARG	A	43	-10.338	70.137	27.642	1.00	75.35	A
ATOM	358	CA	ARG	A	43	-10.202	71.377	28.413	1.00	76.97	A
ATOM	359	CB	ARG	A	43	-9.391	71.131	29.705	1.00	77.23	A
ATOM	360	CG	ARG	A	43	-10.130	70.250	30.753	1.00	77.83	A
ATOM	361	CD	ARG	A	43	-9.265	69.690	31.889	1.00	76.18	A
ATOM	362	NE	ARG	A	43	-10.053	68.919	32.864	1.00	76.19	A
ATOM	363	CZ	ARG	A	43	-10.933	67.967	32.551	1.00	76.17	A
ATOM	364	NH1	ARG	A	43	-11.153	67.657	31.284	1.00	76.24	A
ATOM	365	NH2	ARG	A	43	-11.605	67.326	33.507	1.00	77.89	A
ATOM	366	C	ARG	A	43	-9.560	72.481	27.570	1.00	79.19	A
ATOM	367	O	ARG	A	43	-10.131	72.882	26.548	1.00	79.42	A
ATOM	368	N	ILE	A	44	-8.381	72.970	27.993	1.00	81.42	A
ATOM	369	CA	ILE	A	44	-7.646	74.059	27.276	1.00	84.32	A
ATOM	370	CB	ILE	A	44	-6.073	73.998	27.495	1.00	84.97	A
ATOM	371	CG2	ILE	A	44	-5.292	74.824	26.419	1.00	85.80	A
ATOM	372	CG1	ILE	A	44	-5.728	74.612	28.829	1.00	85.52	A
ATOM	373	CD1	ILE	A	44	-6.344	76.011	29.055	1.00	87.04	A
ATOM	374	C	ILE	A	44	-7.908	73.987	25.790	1.00	86.80	A
ATOM	375	O	ILE	A	44	-8.577	74.829	25.234	1.00	87.60	A
ATOM	376	N	LEU	A	45	-7.318	73.007	25.145	1.00	87.99	A
ATOM	377	CA	LEU	A	45	-7.541	72.910	23.737	1.00	88.13	A
ATOM	378	CB	LEU	A	45	-6.257	72.509	23.009	1.00	88.79	A
ATOM	379	CG	LEU	A	45	-5.940	73.339	21.770	1.00	90.46	A
ATOM	380	CD1	LEU	A	45	-7.147	73.370	20.837	1.00	91.58	A
ATOM	381	CD2	LEU	A	45	-5.596	74.779	22.173	1.00	90.84	A
ATOM	382	C	LEU	A	45	-8.656	71.944	23.376	1.00	88.30	A
ATOM	383	O	LEU	A	45	-9.507	71.665	24.291	1.00	87.82	A
ATOM	384	NT	LEU	A	45	-8.614	71.561	22.151	1.00	88.77	A
ATOM	385	CA	ACE	B	0	29.175	18.175	21.874	1.00	35.90	B
ATOM	386	C	ACE	B	0	27.867	18.849	22.146	1.00	36.69	B
ATOM	387	O	ACE	B	0	27.836	20.078	22.299	1.00	33.24	B
ATOM	388	N	ARG	B	1	26.771	18.065	22.218	1.00	32.69	B
ATOM	389	CA	ARG	B	1	25.440	18.590	22.450	1.00	34.24	B
ATOM	390	CB	ARG	B	1	24.436	17.446	22.644	1.00	33.49	B
ATOM	391	CG	ARG	B	1	22.976	17.878	22.651	1.00	32.92	B
ATOM	392	CD	ARG	B	1	22.436	18.177	21.260	1.00	34.95	B
ATOM	393	NE	ARG	B	1	22.366	16.972	20.443	1.00	38.88	B
ATOM	394	CZ	ARG	B	1	21.548	15.952	20.706	1.00	42.79	B
ATOM	395	NH1	ARG	B	1	20.740	16.012	21.765	1.00	44.66	B
ATOM	396	NH2	ARG	B	1	21.550	14.868	19.943	1.00	39.72	B
ATOM	397	C	ARG	B	1	25.424	19.498	23.685	1.00	35.96	B
ATOM	398	O	ARG	B	1	24.920	20.617	23.628	1.00	36.55	B
ATOM	399	N	MET	B	2	26.008	19.009	24.779	1.00	39.89	B
ATOM	400	CA	MET	B	2	26.077	19.769	26.022	1.00	43.08	B
ATOM	401	CB	MET	B	2	27.113	19.163	26.972	1.00	43.87	B
ATOM	402	CG	MET	B	2	26.728	17.847	27.623	1.00	46.86	B
ATOM	403	SD	MET	B	2	25.304	18.010	28.700	1.00	52.01	B
ATOM	404	CE	MET	B	2	24.024	18.375	27.524	1.00	52.70	B
ATOM	405	C	MET	B	2	26.440	21.219	25.789	1.00	45.76	B
ATOM	406	O	MET	B	2	25.723	22.121	26.212	1.00	44.09	B
ATOM	407	N	LYS	B	3	27.570	21.414	25.125	1.00	47.94	B
ATOM	408	CA	LYS	B	3	28.082	22.736	24.820	1.00	52.42	B
ATOM	409	CB	LYS	B	3	29.455	22.565	24.151	1.00	54.64	B
ATOM	410	CG	LYS	B	3	30.552	23.540	24.595	1.00	58.36	B
ATOM	411	CD	LYS	B	3	30.382	24.937	24.030	1.00	60.17	B

Figure 11G

ATOM	412	CE	LYS	B	3	31.618	25.777	24.321	1.00	62.06	B
ATOM	413	NZ	LYS	B	3	31.561	27.140	23.704	1.00	63.71	B
ATOM	414	C	LYS	B	3	27.095	23.479	23.907	1.00	52.65	B
ATOM	415	O	LYS	B	3	26.858	24.671	24.092	1.00	52.44	B
ATOM	416	N	GLN	B	4	26.517	22.774	22.934	1.00	52.13	B
ATOM	417	CA	GLN	B	4	25.549	23.387	22.032	1.00	54.04	B
ATOM	418	CB	GLN	B	4	24.930	22.330	21.105	1.00	57.72	B
ATOM	419	CG	GLN	B	4	25.792	21.880	19.925	1.00	60.44	B
ATOM	420	CD	GLN	B	4	25.855	22.923	18.816	1.00	62.71	B
ATOM	421	OE1	GLN	B	4	26.404	24.017	18.997	1.00	64.51	B
ATOM	422	NE2	GLN	B	4	25.276	22.592	17.661	1.00	62.62	B
ATOM	423	C	GLN	B	4	24.441	24.062	22.836	1.00	52.63	B
ATOM	424	O	GLN	B	4	24.013	25.162	22.518	1.00	53.56	B
ATOM	425	N	ILE	B	5	23.982	23.379	23.878	1.00	52.62	B
ATOM	426	CA	ILE	B	5	22.929	23.880	24.758	1.00	52.43	B
ATOM	427	CB	ILE	B	5	22.443	22.766	25.721	1.00	51.17	B
ATOM	428	CG2	ILE	B	5	21.412	23.329	26.691	1.00	52.10	B
ATOM	429	CG1	ILE	B	5	21.871	21.592	24.917	1.00	52.55	B
ATOM	430	CD1	ILE	B	5	21.496	20.363	25.754	1.00	53.47	B
ATOM	431	C	ILE	B	5	23.452	25.043	25.600	1.00	53.54	B
ATOM	432	O	ILE	B	5	22.743	26.013	25.849	1.00	52.58	B
ATOM	433	N	GLU	B	6	24.701	24.932	26.036	1.00	55.54	B
ATOM	434	CA	GLU	B	6	25.309	25.970	26.850	1.00	56.11	B
ATOM	435	CB	GLU	B	6	26.637	25.477	27.437	1.00	53.75	B
ATOM	436	CG	GLU	B	6	26.487	24.157	28.171	1.00	53.07	B
ATOM	437	CD	GLU	B	6	27.729	23.735	28.939	1.00	50.56	B
ATOM	438	OE1	GLU	B	6	28.816	23.611	28.329	1.00	49.24	B
ATOM	439	OE2	GLU	B	6	27.604	23.516	30.159	1.00	47.31	B
ATOM	440	C	GLU	B	6	25.522	27.217	26.009	1.00	57.04	B
ATOM	441	O	GLU	B	6	25.418	28.335	26.515	1.00	58.94	B
ATOM	442	N	ASP	B	7	25.811	27.031	24.725	1.00	57.18	B
ATOM	443	CA	ASP	B	7	26.003	28.179	23.848	1.00	58.51	B
ATOM	444	CB	ASP	B	7	26.681	27.772	22.536	1.00	59.88	B
ATOM	445	CG	ASP	B	7	28.121	27.339	22.732	1.00	62.42	B
ATOM	446	OD1	ASP	B	7	28.827	27.979	23.542	1.00	62.53	B
ATOM	447	OD2	ASP	B	7	28.559	26.382	22.056	1.00	66.19	B
ATOM	448	C	ASP	B	7	24.668	28.858	23.543	1.00	58.25	B
ATOM	449	O	ASP	B	7	24.624	30.070	23.314	1.00	56.00	B
ATOM	450	N	LYS	B	8	23.591	28.069	23.547	1.00	57.96	B
ATOM	451	CA	LYS	B	8	22.240	28.563	23.276	1.00	57.58	B
ATOM	452	CB	LYS	B	8	21.331	27.405	22.838	1.00	57.99	B
ATOM	453	CG	LYS	B	8	19.911	27.844	22.484	1.00	60.08	B
ATOM	454	CD	LYS	B	8	19.915	28.785	21.280	1.00	60.12	B
ATOM	455	CE	LYS	B	8	18.697	29.725	21.268	1.00	60.76	B
ATOM	456	NZ	LYS	B	8	17.371	29.062	21.146	1.00	58.46	B
ATOM	457	C	LYS	B	8	21.653	29.248	24.517	1.00	56.86	B
ATOM	458	O	LYS	B	8	20.832	30.166	24.411	1.00	53.70	B
ATOM	459	N	ILE	B	9	22.077	28.790	25.689	1.00	57.87	B
ATOM	460	CA	ILE	B	9	21.621	29.368	26.947	1.00	59.31	B
ATOM	461	CB	ILE	B	9	22.073	28.517	28.161	1.00	57.40	B
ATOM	462	CG2	ILE	B	9	21.788	29.270	29.459	1.00	57.21	B
ATOM	463	CG1	ILE	B	9	21.361	27.165	28.154	1.00	56.21	B
ATOM	464	CD1	ILE	B	9	21.885	26.199	29.212	1.00	54.49	B
ATOM	465	C	ILE	B	9	22.216	30.770	27.093	1.00	60.74	B
ATOM	466	O	ILE	B	9	21.565	31.682	27.608	1.00	61.51	B
ATOM	467	N	GLU	B	10	23.456	30.923	26.633	1.00	61.69	B
ATOM	468	CA	GLU	B	10	24.170	32.198	26.691	1.00	63.76	B
ATOM	469	CB	GLU	B	10	25.629	32.000	26.279	1.00	63.63	B
ATOM	470	CG	GLU	B	10	26.456	33.275	26.254	1.00	65.58	B
ATOM	471	CD	GLU	B	10	27.854	33.054	25.707	1.00	66.48	B

Figure 11H

ATOM	472	OE1	GLU	B	10	27.979	32.751	24.499	1.00	67.38	B
ATOM	473	OE2	GLU	B	10	28.824	33.173	26.485	1.00	66.28	B
ATOM	474	C	GLU	B	10	23.515	33.211	25.757	1.00	65.16	B
ATOM	475	O	GLU	B	10	23.261	34.351	26.141	1.00	65.81	B
ATOM	476	N	GLU	B	11	23.255	32.785	24.524	1.00	66.64	B
ATOM	477	CA	GLU	B	11	22.617	33.637	23.529	1.00	67.59	B
ATOM	478	CB	GLU	B	11	22.348	32.832	22.252	1.00	68.72	B
ATOM	479	CG	GLU	B	11	21.735	33.636	21.117	1.00	72.88	B
ATOM	480	CD	GLU	B	11	22.556	34.864	20.767	1.00	74.80	B
ATOM	481	OE1	GLU	B	11	23.775	34.717	20.526	1.00	75.81	B
ATOM	482	OE2	GLU	B	11	21.978	35.975	20.731	1.00	74.99	B
ATOM	483	C	GLU	B	11	21.307	34.197	24.098	1.00	67.17	B
ATOM	484	O	GLU	B	11	20.998	35.381	23.918	1.00	68.06	B
ATOM	485	N	ILE	B	12	20.541	33.348	24.784	1.00	64.61	B
ATOM	486	CA	ILE	B	12	19.288	33.790	25.389	1.00	61.65	B
ATOM	487	CB	ILE	B	12	18.458	32.600	25.926	1.00	62.84	B
ATOM	488	CG2	ILE	B	12	17.416	33.094	26.940	1.00	62.63	B
ATOM	489	CG1	ILE	B	12	17.799	31.864	24.750	1.00	62.13	B
ATOM	490	CD1	ILE	B	12	16.910	30.698	25.156	1.00	61.39	B
ATOM	491	C	ILE	B	12	19.553	34.776	26.522	1.00	58.17	B
ATOM	492	O	ILE	B	12	19.010	35.881	26.523	1.00	55.05	B
ATOM	493	N	GLU	B	13	20.388	34.384	27.479	1.00	55.87	B
ATOM	494	CA	GLU	B	13	20.710	35.268	28.600	1.00	54.71	B
ATOM	495	CB	GLU	B	13	21.817	34.669	29.477	1.00	50.19	B
ATOM	496	CG	GLU	B	13	21.447	33.331	30.109	1.00	49.30	B
ATOM	497	CD	GLU	B	13	22.577	32.729	30.933	1.00	49.10	B
ATOM	498	OE1	GLU	B	13	23.741	32.765	30.472	1.00	50.79	B
ATOM	499	OE2	GLU	B	13	22.304	32.194	32.027	1.00	47.00	B
ATOM	500	C	GLU	B	13	21.166	36.612	28.047	1.00	55.57	B
ATOM	501	O	GLU	B	13	20.790	37.667	28.557	1.00	56.33	B
ATOM	502	N	SER	B	14	21.950	36.559	26.977	1.00	56.02	B
ATOM	503	CA	SER	B	14	22.468	37.763	26.350	1.00	55.71	B
ATOM	504	CB	SER	B	14	23.488	37.389	25.278	1.00	54.62	B
ATOM	505	CG	SER	B	14	23.968	38.550	24.629	1.00	56.74	B
ATOM	506	C	SER	B	14	21.366	38.624	25.736	1.00	55.96	B
ATOM	507	O	SER	B	14	21.469	39.854	25.696	1.00	54.91	B
ATOM	508	N	LYS	B	15	20.310	37.979	25.263	1.00	55.94	B
ATOM	509	CA	LYS	B	15	19.208	38.704	24.650	1.00	56.72	B
ATOM	510	CB	LYS	B	15	18.454	37.779	23.693	1.00	55.67	B
ATOM	511	CG	LYS	B	15	17.494	38.484	22.772	1.00	58.33	B
ATOM	512	CD	LYS	B	15	17.000	37.527	21.705	1.00	59.89	B
ATOM	513	CE	LYS	B	15	16.440	38.282	20.518	1.00	60.44	B
ATOM	514	NZ	LYS	B	15	16.020	37.375	19.412	1.00	63.67	B
ATOM	515	C	LYS	B	15	18.282	39.207	25.748	1.00	56.31	B
ATOM	516	O	LYS	B	15	17.716	40.296	25.661	1.00	56.65	B
ATOM	517	N	GLN	B	16	18.146	38.403	26.791	1.00	56.76	B
ATOM	518	CA	GLN	B	16	17.293	38.748	27.911	1.00	57.28	B
ATOM	519	CB	GLN	B	16	17.306	37.604	28.923	1.00	56.94	B
ATOM	520	CG	GLN	B	16	16.000	37.394	29.652	1.00	55.90	B
ATOM	521	CD	GLN	B	16	15.908	36.017	30.300	1.00	56.24	B
ATOM	522	OE1	GLN	B	16	16.613	35.722	31.263	1.00	57.78	B
ATOM	523	NE2	GLN	B	16	15.044	35.160	29.760	1.00	55.69	B
ATOM	524	C	GLN	B	16	17.825	40.040	28.528	1.00	58.82	B
ATOM	525	O	GLN	B	16	17.049	40.929	28.905	1.00	59.68	B
ATOM	526	N	LYS	B	17	19.148	40.163	28.621	1.00	59.44	B
ATOM	527	CA	LYS	B	17	19.711	41.379	29.189	1.00	59.84	B
ATOM	528	CB	LYS	B	17	21.228	41.275	29.386	1.00	60.80	B
ATOM	529	CG	LYS	B	17	21.740	42.343	30.356	1.00	64.52	B
ATOM	530	CD	LYS	B	17	23.250	42.325	30.576	1.00	65.30	B
ATOM	531	CE	LYS	B	17	24.008	42.784	29.344	1.00	67.22	B

Figure III

ATOM	532	NZ	LYS	B	17	25.465	42.963	29.625	1.00	67.09	B
ATOM	533	C	LYS	B	17	19.389	42.522	28.230	1.00	59.16	B
ATOM	534	O	LYS	B	17	19.088	43.634	28.656	1.00	55.77	B
ATOM	535	N	LYS	B	18	19.433	42.233	26.931	1.00	58.38	B
ATOM	536	CA	LYS	B	18	19.128	43.248	25.931	1.00	58.35	B
ATOM	537	CB	LYS	B	18	19.247	42.675	24.511	1.00	59.38	B
ATOM	538	CG	LYS	B	18	20.617	42.083	24.130	1.00	61.47	B
ATOM	539	CD	LYS	B	18	21.768	43.111	24.099	1.00	61.91	B
ATOM	540	CE	LYS	B	18	22.034	43.761	25.461	1.00	63.50	B
ATOM	541	NZ	LYS	B	18	23.248	44.620	25.423	1.00	63.66	B
ATOM	542	C	LYS	B	18	17.706	43.761	26.163	1.00	58.27	B
ATOM	543	O	LYS	B	18	17.475	44.969	26.254	1.00	58.82	B
ATOM	544	N	ILE	B	19	16.757	42.835	26.268	1.00	56.89	B
ATOM	545	CA	ILE	B	19	15.356	43.189	26.488	1.00	53.76	B
ATOM	546	CB	ILE	B	19	14.455	41.931	26.488	1.00	53.33	B
ATOM	547	CG2	ILE	B	19	13.057	42.286	26.976	1.00	52.66	B
ATOM	548	CG1	ILE	B	19	14.416	41.322	25.081	1.00	52.79	B
ATOM	549	CD1	ILE	B	19	13.543	40.069	24.970	1.00	54.45	B
ATOM	550	C	ILE	B	19	15.117	43.961	27.786	1.00	52.88	B
ATOM	551	O	ILE	B	19	14.327	44.897	27.809	1.00	51.74	B
ATOM	552	N	GLU	B	20	15.781	43.565	28.869	1.00	51.04	B
ATOM	553	CA	GLU	B	20	15.601	44.267	30.128	1.00	50.08	B
ATOM	554	CB	GLU	B	20	16.403	43.613	31.253	1.00	49.90	B
ATOM	555	CG	GLU	B	20	15.969	42.207	31.584	1.00	54.19	B
ATOM	556	CD	GLU	B	20	16.761	41.620	32.736	1.00	55.98	B
ATOM	557	OE1	GLU	B	20	18.010	41.568	32.641	1.00	53.23	B
ATOM	558	OE2	GLU	B	20	16.127	41.215	33.735	1.00	56.20	B
ATOM	559	C	GLU	B	20	16.053	45.706	29.965	1.00	49.26	B
ATOM	560	O	GLU	B	20	15.479	46.611	30.561	1.00	48.88	B
ATOM	561	N	ASN	B	21	17.093	45.912	29.163	1.00	49.15	B
ATOM	562	CA	ASN	B	21	17.596	47.256	28.930	1.00	49.99	B
ATOM	563	CB	ASN	B	21	18.885	47.229	28.098	1.00	51.35	B
ATOM	564	CG	ASN	B	21	20.054	46.576	28.834	1.00	54.79	B
ATOM	565	OD1	ASN	B	21	20.421	46.978	29.943	1.00	55.96	B
ATOM	566	ND2	ASN	B	21	20.656	45.572	28.205	1.00	57.15	B
ATOM	567	C	ASN	B	21	16.537	48.078	28.202	1.00	49.83	B
ATOM	568	O	ASN	B	21	16.249	49.209	28.591	1.00	50.14	B
ATOM	569	N	GLU	B	22	15.957	47.497	27.153	1.00	47.34	B
ATOM	570	CA	GLU	B	22	14.942	48.160	26.354	1.00	44.99	B
ATOM	571	CB	GLU	B	22	14.534	47.272	25.174	1.00	44.99	B
ATOM	572	CG	GLU	B	22	13.703	47.990	24.116	1.00	51.85	B
ATOM	573	CD	GLU	B	22	14.377	49.268	23.621	1.00	54.71	B
ATOM	574	OE1	GLU	B	22	15.543	49.191	23.182	1.00	55.60	B
ATOM	575	OE2	GLU	B	22	13.743	50.350	23.673	1.00	57.01	B
ATOM	576	C	GLU	B	22	13.710	48.521	27.183	1.00	44.17	B
ATOM	577	O	GLU	B	22	13.044	49.527	26.916	1.00	45.50	B
ATOM	578	N	ILE	B	23	13.386	47.693	28.169	1.00	42.28	B
ATOM	579	CA	ILE	B	23	12.241	47.977	29.024	1.00	40.61	B
ATOM	580	CB	ILE	B	23	11.801	46.724	29.809	1.00	38.57	B
ATOM	581	CG2	ILE	B	23	10.836	47.096	30.925	1.00	37.31	B
ATOM	582	CG1	ILE	B	23	11.138	45.733	28.850	1.00	38.28	B
ATOM	583	CD1	ILE	B	23	10.634	44.436	29.530	1.00	38.32	B
ATOM	584	C	ILE	B	23	12.626	49.108	29.974	1.00	41.50	B
ATOM	585	O	ILE	B	23	11.793	49.926	30.349	1.00	41.54	B
ATOM	586	N	ALA	B	24	13.898	49.170	30.348	1.00	40.42	B
ATOM	587	CA	ALA	B	24	14.349	50.240	31.224	1.00	38.49	B
ATOM	588	CB	ALA	B	24	15.811	50.059	31.578	1.00	34.26	B
ATOM	589	C	ALA	B	24	14.147	51.562	30.490	1.00	37.76	B
ATOM	590	O	ALA	B	24	13.674	52.528	31.078	1.00	38.39	B
ATOM	591	N	ARG	B	25	14.498	51.591	29.204	1.00	36.67	B

Figure 11J

ATOM	592	CA	ARG	B	25	14.354	52.796	28.394	1.00	38.10	B
ATOM	593	CB	ARG	B	25	15.086	52.644	27.051	1.00	40.70	B
ATOM	594	CG	ARG	B	25	16.609	52.668	27.195	1.00	46.74	B
ATOM	595	CD	ARG	B	25	17.315	52.949	25.879	1.00	51.86	B
ATOM	596	NE	ARG	B	25	17.268	51.823	24.954	1.00	56.83	B
ATOM	597	CZ	ARG	B	25	17.894	50.666	25.152	1.00	59.56	B
ATOM	598	NH1	ARG	B	25	18.615	50.477	26.253	1.00	60.08	B
ATOM	599	NH2	ARG	B	25	17.792	49.696	24.257	1.00	59.81	B
ATOM	600	C	ARG	B	25	12.901	53.185	28.158	1.00	36.71	B
ATOM	601	O	ARG	B	25	12.555	54.361	28.165	1.00	36.54	B
ATOM	602	N	ILE	B	26	12.051	52.197	27.942	1.00	36.23	B
ATOM	603	CA	ILE	B	26	10.642	52.454	27.733	1.00	34.33	B
ATOM	604	CB	ILE	B	26	9.944	51.152	27.370	1.00	34.16	B
ATOM	605	CG2	ILE	B	26	8.432	51.293	27.496	1.00	31.45	B
ATOM	606	CG1	ILE	B	26	10.423	50.722	25.985	1.00	34.01	B
ATOM	607	CD1	ILE	B	26	9.879	49.403	25.540	1.00	34.37	B
ATOM	608	C	ILE	B	26	10.046	53.059	29.005	1.00	34.32	B
ATOM	609	O	ILE	B	26	9.317	54.053	28.956	1.00	33.13	B
ATOM	610	N	LYS	B	27	10.371	52.457	30.141	1.00	34.59	B
ATOM	611	CA	LYS	B	27	9.898	52.941	31.433	1.00	35.31	B
ATOM	612	CB	LYS	B	27	10.366	52.005	32.544	1.00	36.43	B
ATOM	613	CG	LYS	B	27	9.398	50.872	32.885	1.00	40.24	B
ATOM	614	CD	LYS	B	27	10.162	49.643	33.347	1.00	44.60	B
ATOM	615	CE	LYS	B	27	11.278	49.991	34.334	1.00	50.65	B
ATOM	616	NZ	LYS	B	27	12.209	48.831	34.560	1.00	54.97	B
ATOM	617	C	LYS	B	27	10.382	54.355	31.712	1.00	35.58	B
ATOM	618	O	LYS	B	27	9.666	55.140	32.318	1.00	36.82	B
ATOM	619	N	LYS	B	28	11.599	54.670	31.268	1.00	36.91	B
ATOM	620	CA	LYS	B	28	12.189	55.993	31.463	1.00	37.71	B
ATOM	621	CB	LYS	B	28	13.627	56.017	30.958	1.00	42.60	B
ATOM	622	CG	LYS	B	28	14.604	56.755	31.851	1.00	49.26	B
ATOM	623	CD	LYS	B	28	15.299	55.778	32.818	1.00	55.52	B
ATOM	624	CE	LYS	B	28	14.318	54.979	33.680	1.00	58.79	B
ATOM	625	NZ	LYS	B	28	15.015	53.887	34.421	1.00	59.10	B
ATOM	626	C	LYS	B	28	11.397	57.044	30.677	1.00	37.60	B
ATOM	627	O	LYS	B	28	10.956	58.045	31.240	1.00	40.12	B
ATOM	628	N	LEU	B	29	11.250	56.826	29.368	1.00	35.33	B
ATOM	629	CA	LEU	B	29	10.515	57.754	28.524	1.00	35.90	B
ATOM	630	CB	LEU	B	29	10.440	57.267	27.071	1.00	36.49	B
ATOM	631	CG	LEU	B	29	9.495	58.127	26.202	1.00	37.58	B
ATOM	632	CD1	LEU	B	29	9.958	59.581	26.260	1.00	36.39	B
ATOM	633	CD2	LEU	B	29	9.441	57.641	24.744	1.00	35.00	B
ATOM	634	C	LEU	B	29	9.103	57.912	29.047	1.00	35.09	B
ATOM	635	O	LEU	B	29	8.568	59.015	29.095	1.00	34.76	B
ATOM	636	N	LEU	B	30	8.512	56.787	29.426	1.00	33.74	B
ATOM	637	CA	LEU	B	30	7.161	56.746	29.946	1.00	30.65	B
ATOM	638	CB	LEU	B	30	6.789	55.284	30.181	1.00	32.72	B
ATOM	639	CG	LEU	B	30	5.385	54.822	30.558	1.00	31.65	B
ATOM	640	CD1	LEU	B	30	4.353	55.404	29.599	1.00	34.37	B
ATOM	641	CD2	LEU	B	30	5.377	53.282	30.511	1.00	31.69	B
ATOM	642	C	LEU	B	30	6.985	57.588	31.213	1.00	31.56	B
ATOM	643	O	LEU	B	30	6.051	58.398	31.301	1.00	26.54	B
ATOM	644	N	GLN	B	31	7.860	57.442	32.206	1.00	31.24	B
ATOM	645	CA	GLN	B	31	7.668	58.265	33.398	1.00	33.01	B
ATOM	646	CB	GLN	B	31	8.551	57.801	34.564	1.00	33.79	B
ATOM	647	CG	GLN	B	31	10.013	57.729	34.321	1.00	40.81	B
ATOM	648	CD	GLN	B	31	10.737	57.086	35.491	1.00	44.04	B
ATOM	649	OE1	GLN	B	31	10.804	57.648	36.598	1.00	43.99	B
ATOM	650	NE2	GLN	B	31	11.270	55.889	35.258	1.00	41.97	B
ATOM	651	C	GLN	B	31	7.906	59.734	33.072	1.00	34.12	B

Figure 11K

ATOM	652	O	GLN	B	31	7.420	60.636	33.766	1.00	30.63	B
ATOM	653	N	LEU	B	32	8.629	59.961	31.984	1.00	34.46	B
ATOM	654	CA	LEU	B	32	8.935	61.292	31.523	1.00	36.10	B
ATOM	655	CB	LEU	B	32	10.070	61.231	30.504	1.00	40.01	B
ATOM	656	CG	LEU	B	32	10.340	62.546	29.775	1.00	40.15	B
ATOM	657	CD1	LEU	B	32	10.853	63.586	30.765	1.00	43.23	B
ATOM	658	CD2	LEU	B	32	11.354	62.310	28.668	1.00	43.00	B
ATOM	659	C	LEU	B	32	7.711	61.949	30.890	1.00	36.08	B
ATOM	660	O	LEU	B	32	7.552	63.162	30.964	1.00	37.71	B
ATOM	661	N	THR	B	33	6.859	61.149	30.255	1.00	32.40	B
ATOM	662	CA	THR	B	33	5.659	61.679	29.617	1.00	31.31	B
ATOM	663	CB	THR	B	33	5.179	60.753	28.480	1.00	30.70	B
ATOM	664	OG1	THR	B	33	4.536	59.603	29.030	1.00	40.03	B
ATOM	665	CG2	THR	B	33	6.371	60.282	27.654	1.00	31.28	B
ATOM	666	C	THR	B	33	4.550	61.845	30.668	1.00	30.03	B
ATOM	667	O	THR	B	33	3.739	62.772	30.585	1.00	30.10	B
ATOM	668	N	VAL	B	34	4.507	60.933	31.636	1.00	27.29	B
ATOM	669	CA	VAL	B	34	3.546	61.010	32.735	1.00	25.28	B
ATOM	670	CB	VAL	B	34	3.695	59.806	33.690	1.00	26.71	B
ATOM	671	CG1	VAL	B	34	2.920	60.036	34.985	1.00	27.25	B
ATOM	672	CG2	VAL	B	34	3.176	58.565	32.997	1.00	23.84	B
ATOM	673	C	VAL	B	34	3.822	62.310	33.476	1.00	22.65	B
ATOM	674	O	VAL	B	34	2.899	63.064	33.763	1.00	21.36	B
ATOM	675	N	TRP	B	35	5.100	62.580	33.757	1.00	22.24	B
ATOM	676	CA	TRP	B	35	5.502	63.828	34.414	1.00	20.87	B
ATOM	677	CB	TRP	B	35	7.016	63.843	34.653	1.00	23.71	B
ATOM	678	CG	TRP	B	35	7.523	65.040	35.434	1.00	26.08	B
ATOM	679	CD2	TRP	B	35	7.013	65.551	36.681	1.00	25.13	B
ATOM	680	CE2	TRP	B	35	7.767	66.698	37.003	1.00	28.35	B
ATOM	681	CE3	TRP	B	35	5.985	65.143	37.547	1.00	24.83	B
ATOM	682	CD1	TRP	B	35	8.540	65.880	35.074	1.00	25.67	B
ATOM	683	NE1	TRP	B	35	8.692	66.877	36.006	1.00	27.74	B
ATOM	684	CZ2	TRP	B	35	7.532	67.455	38.165	1.00	28.38	B
ATOM	685	CZ3	TRP	B	35	5.749	65.889	38.699	1.00	23.47	B
ATOM	686	CH2	TRP	B	35	6.516	67.034	38.999	1.00	28.31	B
ATOM	687	C	TRP	B	35	5.121	65.039	33.564	1.00	24.26	B
ATOM	688	O	TRP	B	35	4.695	66.063	34.088	1.00	23.94	B
ATOM	689	N	GLY	B	36	5.308	64.927	32.247	1.00	25.59	B
ATOM	690	CA	GLY	B	36	4.961	66.013	31.348	1.00	23.22	B
ATOM	691	C	GLY	B	36	3.479	66.364	31.343	1.00	25.72	B
ATOM	692	O	GLY	B	36	3.138	67.539	31.352	1.00	28.94	B
ATOM	693	N	ILE	B	37	2.610	65.356	31.311	1.00	27.20	B
ATOM	694	CA	ILE	B	37	1.160	65.560	31.315	1.00	24.67	B
ATOM	695	CB	ILE	B	37	0.429	64.223	31.230	1.00	24.72	B
ATOM	696	CG2	ILE	B	37	-1.085	64.410	31.416	1.00	29.15	B
ATOM	697	CG1	ILE	B	37	0.700	63.581	29.879	1.00	22.40	B
ATOM	698	CD1	ILE	B	37	0.023	62.237	29.714	1.00	24.46	B
ATOM	699	C	ILE	B	37	0.734	66.295	32.579	1.00	25.86	B
ATOM	700	O	ILE	B	37	-0.019	67.255	32.517	1.00	25.23	B
ATOM	701	N	LYS	B	38	1.242	65.840	33.722	1.00	26.17	B
ATOM	702	CA	LYS	B	38	0.967	66.449	35.020	1.00	22.96	B
ATOM	703	CB	LYS	B	38	1.656	65.652	36.130	1.00	22.07	B
ATOM	704	CG	LYS	B	38	0.953	64.410	36.522	1.00	25.14	B
ATOM	705	CD	LYS	B	38	-0.225	64.727	37.423	1.00	28.48	B
ATOM	706	CE	LYS	B	38	-1.014	63.468	37.617	1.00	28.77	B
ATOM	707	NZ	LYS	B	38	-1.331	62.953	36.269	1.00	34.06	B
ATOM	708	C	LYS	B	38	1.458	67.877	35.102	1.00	23.87	B
ATOM	709	O	LYS	B	38	0.770	68.736	35.640	1.00	20.93	B
ATOM	710	N	GLN	B	39	2.662	68.140	34.593	1.00	26.53	B
ATOM	711	CA	GLN	B	39	3.189	69.493	34.682	1.00	30.76	B

Figure 11L

ATOM	712	CB	GLN	B	39	4.629	69.583	34.197	1.00	33.05	B
ATOM	713	CG	GLN	B	39	5.436	70.614	34.985	1.00	43.49	B
ATOM	714	CD	GLN	B	39	4.822	72.026	35.008	1.00	48.65	B
ATOM	715	OE1	GLN	B	39	4.889	72.774	34.021	1.00	51.46	B
ATOM	716	NE2	GLN	B	39	4.220	72.389	36.143	1.00	47.35	B
ATOM	717	C	GLN	B	39	2.343	70.417	33.843	1.00	31.81	B
ATOM	718	O	GLN	B	39	2.125	71.574	34.206	1.00	31.08	B
ATOM	719	N	LEU	B	40	1.897	69.904	32.703	1.00	31.01	B
ATOM	720	CA	LEU	B	40	1.065	70.671	31.807	1.00	33.41	B
ATOM	721	CB	LEU	B	40	0.872	69.886	30.517	1.00	32.63	B
ATOM	722	CG	LEU	B	40	-0.126	70.405	29.482	1.00	34.65	B
ATOM	723	CD1	LEU	B	40	0.171	71.843	29.092	1.00	35.24	B
ATOM	724	CD2	LEU	B	40	-0.058	69.495	28.281	1.00	35.90	B
ATOM	725	C	LEU	B	40	-0.289	70.943	32.469	1.00	36.85	B
ATOM	726	O	LEU	B	40	-0.874	72.010	32.314	1.00	37.81	B
ATOM	727	N	GLN	B	41	-0.768	69.964	33.215	1.00	36.13	B
ATOM	728	CA	GLN	B	41	-2.046	70.063	33.894	1.00	37.74	B
ATOM	729	CB	GLN	B	41	-2.369	68.718	34.517	1.00	41.31	B
ATOM	730	CG	GLN	B	41	-3.833	68.459	34.735	1.00	47.08	B
ATOM	731	CD	GLN	B	41	-4.070	67.139	35.420	1.00	54.09	B
ATOM	732	OE1	GLN	B	41	-3.517	66.102	35.013	1.00	55.42	B
ATOM	733	NE2	GLN	B	41	-4.908	67.154	36.461	1.00	54.90	B
ATOM	734	C	GLN	B	41	-2.039	71.148	34.974	1.00	39.95	B
ATOM	735	O	GLN	B	41	-2.988	71.925	35.089	1.00	39.23	B
ATOM	736	N	ALA	B	42	-0.972	71.194	35.767	1.00	39.05	B
ATOM	737	CA	ALA	B	42	-0.845	72.188	36.824	1.00	38.56	B
ATOM	738	CB	ALA	B	42	0.345	71.852	37.757	1.00	34.14	B
ATOM	739	C	ALA	B	42	-0.647	73.566	36.228	1.00	40.18	B
ATOM	740	O	ALA	B	42	-1.139	74.560	36.765	1.00	41.44	B
ATOM	741	N	ARG	B	43	0.078	73.634	35.118	1.00	41.82	B
ATOM	742	CA	ARG	B	43	0.340	74.910	34.476	1.00	43.71	B
ATOM	743	CB	ARG	B	43	1.242	74.713	33.260	1.00	47.26	B
ATOM	744	CG	ARG	B	43	1.703	75.997	32.592	1.00	51.08	B
ATOM	745	CD	ARG	B	43	2.582	75.677	31.401	1.00	54.95	B
ATOM	746	NE	ARG	B	43	3.778	74.947	31.813	1.00	57.04	B
ATOM	747	CZ	ARG	B	43	4.819	75.499	32.428	1.00	56.95	B
ATOM	748	NH1	ARG	B	43	4.816	76.794	32.703	1.00	55.89	B
ATOM	749	NH2	ARG	B	43	5.858	74.753	32.781	1.00	57.00	B
ATOM	750	C	ARG	B	43	-0.987	75.521	34.048	1.00	42.38	B
ATOM	751	O	ARG	B	43	-1.308	76.657	34.398	1.00	41.41	B
ATOM	752	N	ILE	B	44	-1.756	74.736	33.310	1.00	41.63	B
ATOM	753	CA	ILE	B	44	-3.059	75.143	32.810	1.00	43.24	B
ATOM	754	CB	ILE	B	44	-3.634	74.085	31.866	1.00	44.23	B
ATOM	755	CG2	ILE	B	44	-5.083	74.403	31.592	1.00	45.04	B
ATOM	756	CG1	ILE	B	44	-2.778	73.964	30.600	1.00	47.45	B
ATOM	757	CD1	ILE	B	44	-3.156	72.745	29.719	1.00	49.42	B
ATOM	758	C	ILE	B	44	-4.081	75.306	33.935	1.00	42.37	B
ATOM	759	O	ILE	B	44	-4.422	76.416	34.332	1.00	42.08	B
ATOM	760	N	LEU	B	45	-4.573	74.162	34.398	1.00	42.20	B
ATOM	761	CA	LEU	B	45	-5.564	74.042	35.450	1.00	43.16	B
ATOM	762	CB	LEU	B	45	-6.041	72.592	35.513	1.00	46.08	B
ATOM	763	CG	LEU	B	45	-6.459	72.001	34.162	1.00	47.45	B
ATOM	764	CD1	LEU	B	45	-7.011	70.594	34.357	1.00	47.51	B
ATOM	765	CD2	LEU	B	45	-7.504	72.899	33.521	1.00	48.61	B
ATOM	766	C	LEU	B	45	-5.016	74.467	36.810	1.00	42.48	B
ATOM	767	O	LEU	B	45	-5.674	75.260	37.483	1.00	45.15	B
ATOM	768	WT	LEU	B	45	-3.945	73.987	37.206	1.00	45.66	B
ATOM	769	CA	ACE	C	0	15.143	11.286	26.819	1.00	82.49	C
ATOM	770	C	ACE	C	0	14.856	12.476	27.674	1.00	82.44	C
ATOM	771	O	ACE	C	0	13.700	12.858	27.851	1.00	84.06	C

Figure 11M

ATOM	772	N	ARG	C	1	15.890	13.103	28.220	1.00	82.91	C
ATOM	773	CA	ARG	C	1	15.663	14.253	29.073	1.00	83.87	C
ATOM	774	CB	ARG	C	1	16.156	13.970	30.491	1.00	83.74	C
ATOM	775	CG	ARG	C	1	15.769	15.063	31.456	1.00	83.47	C
ATOM	776	CD	ARG	C	1	14.340	15.542	31.156	1.00	81.66	C
ATOM	777	NE	ARG	C	1	13.249	14.748	31.726	1.00	81.00	C
ATOM	778	CZ	ARG	C	1	13.069	13.434	31.597	1.00	79.16	C
ATOM	779	NH1	ARG	C	1	13.901	12.678	30.889	1.00	79.80	C
ATOM	780	NH2	ARG	C	1	12.010	12.875	32.168	1.00	79.18	C
ATOM	781	C	ARG	C	1	16.282	15.541	28.550	1.00	85.03	C
ATOM	782	O	ARG	C	1	15.975	16.644	29.016	1.00	85.10	C
ATOM	783	N	MET	C	2	17.169	15.394	27.581	1.00	85.40	C
ATOM	784	CA	MET	C	2	17.778	16.568	27.012	1.00	86.91	C
ATOM	785	CB	MET	C	2	19.063	16.215	26.290	1.00	88.20	C
ATOM	786	CG	MET	C	2	19.711	17.410	25.653	1.00	89.72	C
ATOM	787	SD	MET	C	2	21.192	16.917	24.823	1.00	94.98	C
ATOM	788	CE	MET	C	2	22.111	16.349	26.176	1.00	91.53	C
ATOM	789	C	MET	C	2	16.771	17.154	26.036	1.00	87.44	C
ATOM	790	O	MET	C	2	16.699	18.368	25.872	1.00	89.05	C
ATOM	791	N	LYS	C	3	16.001	16.278	25.391	1.00	85.66	C
ATOM	792	CA	LYS	C	3	14.973	16.712	24.444	1.00	83.09	C
ATOM	793	CB	LYS	C	3	14.033	15.551	24.107	1.00	82.50	C
ATOM	794	CG	LYS	C	3	12.921	15.895	23.122	1.00	81.54	C
ATOM	795	CD	LYS	C	3	11.926	14.746	23.005	1.00	81.93	C
ATOM	796	CE	LYS	C	3	10.866	15.022	21.952	1.00	80.79	C
ATOM	797	NZ	LYS	C	3	10.154	16.300	22.214	1.00	82.56	C
ATOM	798	C	LYS	C	3	14.177	17.809	25.128	1.00	82.12	C
ATOM	799	O	LYS	C	3	14.053	18.925	24.617	1.00	81.76	C
ATOM	800	N	GLN	C	4	13.651	17.474	26.302	1.00	80.32	C
ATOM	801	CA	GLN	C	4	12.856	18.401	27.094	1.00	78.87	C
ATOM	802	CB	GLN	C	4	12.504	17.759	28.440	1.00	79.91	C
ATOM	803	CG	GLN	C	4	12.122	16.275	28.356	1.00	80.66	C
ATOM	804	CD	GLN	C	4	11.087	15.971	27.280	1.00	81.02	C
ATOM	805	OE1	GLN	C	4	11.348	16.140	26.082	1.00	79.52	C
ATOM	806	NE2	GLN	C	4	9.907	15.516	27.701	1.00	81.57	C
ATOM	807	C	GLN	C	4	13.667	19.680	27.299	1.00	77.97	C
ATOM	808	O	GLN	C	4	13.186	20.781	27.032	1.00	78.45	C
ATOM	809	N	ILE	C	5	14.902	19.530	27.772	1.00	76.07	C
ATOM	810	CA	ILE	C	5	15.785	20.670	27.974	1.00	73.89	C
ATOM	811	CB	ILE	C	5	17.206	20.220	28.381	1.00	73.07	C
ATOM	812	CG2	ILE	C	5	18.175	21.388	28.264	1.00	71.17	C
ATOM	813	CG1	ILE	C	5	17.174	19.623	29.795	1.00	72.84	C
ATOM	814	CD1	ILE	C	5	18.518	19.113	30.285	1.00	71.39	C
ATOM	815	C	ILE	C	5	15.880	21.423	26.656	1.00	74.14	C
ATOM	816	O	ILE	C	5	15.939	22.651	26.628	1.00	73.70	C
ATOM	817	N	GLU	C	6	15.895	20.664	25.567	1.00	73.88	C
ATOM	818	CA	GLU	C	6	15.972	21.222	24.225	1.00	73.70	C
ATOM	819	CB	GLU	C	6	16.395	20.135	23.229	1.00	72.24	C
ATOM	820	CG	GLU	C	6	17.787	19.535	23.464	1.00	69.96	C
ATOM	821	CD	GLU	C	6	18.922	20.428	22.985	1.00	68.01	C
ATOM	822	OE1	GLU	C	6	19.044	21.575	23.461	1.00	65.93	C
ATOM	823	OE2	GLU	C	6	19.702	19.963	22.125	1.00	68.18	C
ATOM	824	C	GLU	C	6	14.602	21.773	23.842	1.00	74.50	C
ATOM	825	O	GLU	C	6	14.476	22.546	22.890	1.00	75.27	C
ATOM	826	N	ASP	C	7	13.577	21.372	24.587	1.00	74.82	C
ATOM	827	CA	ASP	C	7	12.218	21.838	24.327	1.00	76.17	C
ATOM	828	CB	ASP	C	7	11.195	20.742	24.644	1.00	77.40	C
ATOM	829	CG	ASP	C	7	11.408	19.488	23.818	1.00	78.45	C
ATOM	830	OD1	ASP	C	7	11.518	19.609	22.580	1.00	79.26	C
ATOM	831	OD2	ASP	C	7	11.452	18.380	24.404	1.00	79.10	C

Figure 11N



ATOM	832	C	ASP	C	7	11.906	23.079	25.160	1.00	75.92	C
ATOM	833	O	ASP	C	7	11.379	24.063	24.643	1.00	77.15	C
ATOM	834	N	LYS	C	8	12.223	23.024	26.452	1.00	74.05	C
ATOM	835	CA	LYS	C	8	11.987	24.157	27.336	1.00	71.19	C
ATOM	836	CB	LYS	C	8	12.565	23.886	28.727	1.00	72.69	C
ATOM	837	CG	LYS	C	8	11.647	24.225	29.901	1.00	72.96	C
ATOM	838	CD	LYS	C	8	10.428	23.312	29.921	1.00	75.00	C
ATOM	839	CE	LYS	C	8	9.587	23.471	31.197	1.00	76.69	C
ATOM	840	NZ	LYS	C	8	8.998	24.829	31.389	1.00	73.68	C
ATOM	841	C	LYS	C	8	12.727	25.319	26.679	1.00	69.24	C
ATOM	842	O	LYS	C	8	12.295	26.469	26.745	1.00	69.77	C
ATOM	843	N	ILE	C	9	13.855	25.013	26.046	1.00	65.63	C
ATOM	844	CA	ILE	C	9	14.609	26.053	25.362	1.00	64.27	C
ATOM	845	CB	ILE	C	9	15.950	25.511	24.812	1.00	62.88	C
ATOM	846	CG2	ILE	C	9	16.585	26.515	23.871	1.00	62.42	C
ATOM	847	CG1	ILE	C	9	16.900	25.231	25.976	1.00	64.19	C
ATOM	848	CD1	ILE	C	9	18.244	24.656	25.557	1.00	64.32	C
ATOM	849	C	ILE	C	9	13.756	26.605	24.223	1.00	63.69	C
ATOM	850	O	ILE	C	9	13.735	27.816	23.985	1.00	63.21	C
ATOM	851	N	GLU	C	10	13.036	25.712	23.543	1.00	62.89	C
ATOM	852	CA	GLU	C	10	12.163	26.092	22.429	1.00	62.21	C
ATOM	853	CB	GLU	C	10	11.419	24.865	21.886	1.00	63.68	C
ATOM	854	CG	GLU	C	10	10.451	25.180	20.751	1.00	66.12	C
ATOM	855	CD	GLU	C	10	9.688	23.961	20.251	1.00	67.29	C
ATOM	856	OE1	GLU	C	10	8.874	24.125	19.318	1.00	69.26	C
ATOM	857	OE2	GLU	C	10	9.894	22.845	20.780	1.00	68.71	C
ATOM	858	C	GLU	C	10	11.142	27.147	22.831	1.00	60.65	C
ATOM	859	O	GLU	C	10	10.991	28.157	22.147	1.00	60.16	C
ATOM	860	N	GLU	C	11	10.429	26.898	23.927	1.00	60.41	C
ATOM	861	CA	GLU	C	11	9.415	27.826	24.435	1.00	58.98	C
ATOM	862	CB	GLU	C	11	8.736	27.243	25.683	1.00	59.35	C
ATOM	863	CG	GLU	C	11	9.709	26.588	26.652	1.00	61.78	C
ATOM	864	CD	GLU	C	11	9.376	26.801	28.127	1.00	63.00	C
ATOM	865	OE1	GLU	C	11	9.329	27.972	28.563	1.00	64.82	C
ATOM	866	OE2	GLU	C	11	9.184	25.804	28.855	1.00	60.50	C
ATOM	867	C	GLU	C	11	10.021	29.186	24.772	1.00	58.03	C
ATOM	868	O	GLU	C	11	9.519	30.229	24.351	1.00	59.21	C
ATOM	869	N	ILE	C	12	11.103	29.178	25.532	1.00	56.15	C
ATOM	870	CA	ILE	C	12	11.765	30.415	25.902	1.00	56.41	C
ATOM	871	CB	ILE	C	12	13.043	30.139	26.710	1.00	55.29	C
ATOM	872	CG2	ILE	C	12	13.791	31.448	26.950	1.00	52.26	C
ATOM	873	CG1	ILE	C	12	12.680	29.404	28.008	1.00	55.06	C
ATOM	874	CD1	ILE	C	12	13.858	29.085	28.914	1.00	55.11	C
ATOM	875	C	ILE	C	12	12.132	31.239	24.671	1.00	57.76	C
ATOM	876	O	ILE	C	12	11.944	32.454	24.659	1.00	59.18	C
ATOM	877	N	GLU	C	13	12.668	30.589	23.642	1.00	60.62	C
ATOM	878	CA	GLU	C	13	13.039	31.312	22.423	1.00	62.64	C
ATOM	879	CB	GLU	C	13	13.916	30.449	21.497	1.00	66.02	C
ATOM	880	CG	GLU	C	13	13.319	29.091	21.138	1.00	70.85	C
ATOM	881	CD	GLU	C	13	14.091	28.355	20.041	1.00	73.58	C
ATOM	882	OE1	GLU	C	13	15.330	28.233	20.163	1.00	72.99	C
ATOM	883	OE2	GLU	C	13	13.456	27.887	19.064	1.00	73.78	C
ATOM	884	C	GLU	C	13	11.785	31.748	21.679	1.00	60.74	C
ATOM	885	O	GLU	C	13	11.808	32.733	20.946	1.00	61.13	C
ATOM	886	N	SER	C	14	10.695	31.010	21.864	1.00	59.71	C
ATOM	887	CA	SER	C	14	9.432	31.350	21.211	1.00	60.53	C
ATOM	888	CB	SER	C	14	8.392	30.248	21.439	1.00	59.88	C
ATOM	889	CG	SER	C	14	7.157	30.571	20.820	1.00	56.34	C
ATOM	890	C	SER	C	14	8.921	32.668	21.790	1.00	61.04	C
ATOM	891	O	SER	C	14	8.793	33.655	21.073	1.00	59.08	C

Figure 110

ATOM	892	N	LYS	C	15	8.632	32.671	23.091	1.00	62.79	C
ATOM	893	CA	LYS	C	15	8.153	33.873	23.771	1.00	64.30	C
ATOM	894	CB	LYS	C	15	7.949	33.612	25.273	1.00	65.74	C
ATOM	895	CG	LYS	C	15	6.637	32.903	25.642	1.00	68.25	C
ATOM	896	CD	LYS	C	15	6.534	32.695	27.154	1.00	69.92	C
ATOM	897	CE	LYS	C	15	5.186	32.131	27.564	1.00	70.69	C
ATOM	898	NZ	LYS	C	15	4.078	33.079	27.241	1.00	73.69	C
ATOM	899	C	LYS	C	15	9.130	35.029	23.601	1.00	64.03	C
ATOM	900	O	LYS	C	15	8.723	36.175	23.408	1.00	64.04	C
ATOM	901	N	GLN	C	16	10.418	34.721	23.678	1.00	63.47	C
ATOM	902	CA	GLN	C	16	11.451	35.733	23.537	1.00	65.82	C
ATOM	903	CB	GLN	C	16	12.813	35.064	23.393	1.00	65.17	C
ATOM	904	CG	GLN	C	16	13.970	36.027	23.413	1.00	65.29	C
ATOM	905	CD	GLN	C	16	14.944	35.695	24.516	1.00	66.93	C
ATOM	906	OE1	GLN	C	16	15.940	36.389	24.719	1.00	68.97	C
ATOM	907	NE2	GLN	C	16	14.657	34.621	25.244	1.00	66.55	C
ATOM	908	C	GLN	C	16	11.157	36.605	22.317	1.00	67.53	C
ATOM	909	O	GLN	C	16	11.172	37.836	22.397	1.00	68.90	C
ATOM	910	N	LYS	C	17	10.886	35.952	21.193	1.00	67.63	C
ATOM	911	CA	LYS	C	17	10.566	36.648	19.954	1.00	67.83	C
ATOM	912	CB	LYS	C	17	10.355	35.627	18.833	1.00	69.39	C
ATOM	913	CG	LYS	C	17	9.747	36.199	17.556	1.00	72.05	C
ATOM	914	CD	LYS	C	17	10.657	37.203	16.835	1.00	73.47	C
ATOM	915	CE	LYS	C	17	9.946	37.784	15.613	1.00	74.71	C
ATOM	916	NZ	LYS	C	17	10.885	38.603	14.795	1.00	76.15	C
ATOM	917	C	LYS	C	17	9.306	37.492	20.123	1.00	66.64	C
ATOM	918	O	LYS	C	17	9.244	38.632	19.652	1.00	67.45	C
ATOM	919	N	LYS	C	18	8.300	36.924	20.784	1.00	64.29	C
ATOM	920	CA	LYS	C	18	7.049	37.641	21.019	1.00	63.62	C
ATOM	921	CB	LYS	C	18	5.979	36.719	21.627	1.00	64.15	C
ATOM	922	CG	LYS	C	18	5.088	36.062	20.586	1.00	66.52	C
ATOM	923	CD	LYS	C	18	3.935	35.297	21.220	1.00	68.98	C
ATOM	924	CE	LYS	C	18	4.427	34.076	21.970	1.00	70.96	C
ATOM	925	NZ	LYS	C	18	5.098	33.116	21.040	1.00	72.62	C
ATOM	926	C	LYS	C	18	7.265	38.852	21.922	1.00	61.00	C
ATOM	927	O	LYS	C	18	6.854	39.958	21.585	1.00	61.84	C
ATOM	928	N	ILE	C	19	7.904	38.653	23.067	1.00	56.58	C
ATOM	929	CA	ILE	C	19	8.179	39.765	23.961	1.00	53.92	C
ATOM	930	CB	ILE	C	19	9.101	39.329	25.119	1.00	52.10	C
ATOM	931	CG2	ILE	C	19	9.719	40.545	25.799	1.00	51.95	C
ATOM	932	CG1	ILE	C	19	8.304	38.463	26.095	1.00	51.65	C
ATOM	933	CD1	ILE	C	19	9.103	37.908	27.247	1.00	50.93	C
ATOM	934	C	ILE	C	19	8.833	40.893	23.165	1.00	53.24	C
ATOM	935	O	ILE	C	19	8.604	42.069	23.438	1.00	52.35	C
ATOM	936	N	GLU	C	20	9.642	40.534	22.173	1.00	53.82	C
ATOM	937	CA	GLU	C	20	10.294	41.536	21.338	1.00	54.86	C
ATOM	938	CB	GLU	C	20	11.393	40.910	20.472	1.00	55.74	C
ATOM	939	CG	GLU	C	20	12.554	40.318	21.251	1.00	56.50	C
ATOM	940	CD	GLU	C	20	13.683	39.851	20.352	1.00	56.98	C
ATOM	941	OE1	GLU	C	20	13.473	38.918	19.543	1.00	56.87	C
ATOM	942	OE2	GLU	C	20	14.786	40.427	20.453	1.00	58.79	C
ATOM	943	C	GLU	C	20	9.245	42.188	20.437	1.00	55.80	C
ATOM	944	O	GLU	C	20	9.311	43.382	20.166	1.00	55.44	C
ATOM	945	N	ASN	C	21	8.289	41.389	19.972	1.00	55.46	C
ATOM	946	CA	ASN	C	21	7.223	41.899	19.118	1.00	57.62	C
ATOM	947	CB	ASN	C	21	6.392	40.754	18.530	1.00	59.92	C
ATOM	948	CG	ASN	C	21	7.060	40.101	17.325	1.00	63.29	C
ATOM	949	OD1	ASN	C	21	6.574	39.092	16.806	1.00	62.67	C
ATOM	950	ND2	ASN	C	21	8.169	40.684	16.866	1.00	61.87	C
ATOM	951	C	ASN	C	21	6.307	42.829	19.891	1.00	58.25	C

Figure 11P

ATOM	952	O	ASN	C	21	5.649	43.697	19.309	1.00	59.75	C
ATOM	953	N	GLU	C	22	6.255	42.645	21.206	1.00	56.32	C
ATOM	954	CA	GLU	C	22	5.411	43.489	22.030	1.00	53.64	C
ATOM	955	CB	GLU	C	22	5.014	42.756	23.313	1.00	55.42	C
ATOM	956	CG	GLU	C	22	3.786	43.357	23.967	1.00	60.12	C
ATOM	957	CD	GLU	C	22	2.506	43.082	23.188	1.00	61.82	C
ATOM	958	OE1	GLU	C	22	2.559	43.024	21.942	1.00	62.49	C
ATOM	959	OE2	GLU	C	22	1.435	42.954	23.825	1.00	63.39	C
ATOM	960	C	GLU	C	22	6.158	44.791	22.344	1.00	50.89	C
ATOM	961	O	GLU	C	22	5.573	45.873	22.282	1.00	49.72	C
ATOM	962	N	ILE	C	23	7.448	44.691	22.665	1.00	47.08	C
ATOM	963	CA	ILE	C	23	8.259	45.876	22.948	1.00	46.40	C
ATOM	964	CB	ILE	C	23	9.752	45.504	23.290	1.00	47.53	C
ATOM	965	CG2	ILE	C	23	10.707	46.653	22.910	1.00	44.86	C
ATOM	966	CG1	ILE	C	23	9.898	45.178	24.783	1.00	45.28	C
ATOM	967	CD1	ILE	C	23	9.101	44.004	25.256	1.00	45.91	C
ATOM	968	C	ILE	C	23	8.222	46.771	21.717	1.00	46.76	C
ATOM	969	O	ILE	C	23	8.317	47.999	21.822	1.00	46.87	C
ATOM	970	N	ALA	C	24	8.071	46.137	20.556	1.00	47.50	C
ATOM	971	CA	ALA	C	24	8.002	46.828	19.271	1.00	46.10	C
ATOM	972	CB	ALA	C	24	8.112	45.809	18.126	1.00	44.51	C
ATOM	973	C	ALA	C	24	6.706	47.644	19.137	1.00	45.09	C
ATOM	974	O	ALA	C	24	6.741	48.810	18.752	1.00	43.05	C
ATOM	975	N	ARG	C	25	5.566	47.034	19.445	1.00	43.64	C
ATOM	976	CA	ARG	C	25	4.301	47.753	19.346	1.00	45.79	C
ATOM	977	CB	ARG	C	25	3.115	46.807	19.581	1.00	44.07	C
ATOM	978	CG	ARG	C	25	3.045	45.680	18.564	1.00	48.16	C
ATOM	979	CD	ARG	C	25	1.677	44.986	18.458	1.00	50.13	C
ATOM	980	NE	ARG	C	25	1.216	44.299	19.664	1.00	54.12	C
ATOM	981	CZ	ARG	C	25	0.665	44.888	20.725	1.00	58.36	C
ATOM	982	NH1	ARG	C	25	0.475	46.206	20.756	1.00	59.26	C
ATOM	983	NH2	ARG	C	25	0.268	44.148	21.755	1.00	59.83	C
ATOM	984	C	ARG	C	25	4.257	48.908	20.345	1.00	47.24	C
ATOM	985	O	ARG	C	25	3.941	50.038	19.978	1.00	50.68	C
ATOM	986	N	ILE	C	26	4.584	48.617	21.601	1.00	47.54	C
ATOM	987	CA	ILE	C	26	4.591	49.608	22.673	1.00	44.40	C
ATOM	988	CB	ILE	C	26	5.042	48.959	24.001	1.00	43.91	C
ATOM	989	CG2	ILE	C	26	5.259	50.026	25.071	1.00	45.47	C
ATOM	990	CG1	ILE	C	26	4.010	47.930	24.450	1.00	42.59	C
ATOM	991	CD1	ILE	C	26	4.445	47.138	25.663	1.00	40.19	C
ATOM	992	C	ILE	C	26	5.532	50.766	22.379	1.00	44.58	C
ATOM	993	O	ILE	C	26	5.193	51.935	22.564	1.00	42.04	C
ATOM	994	N	LYS	C	27	6.721	50.422	21.919	1.00	46.75	C
ATOM	995	CA	LYS	C	27	7.754	51.394	21.619	1.00	51.78	C
ATOM	996	CB	LYS	C	27	8.915	50.674	20.951	1.00	54.23	C
ATOM	997	CG	LYS	C	27	10.184	51.465	20.863	1.00	57.21	C
ATOM	998	CD	LYS	C	27	11.313	50.479	20.644	1.00	60.99	C
ATOM	999	CE	LYS	C	27	12.660	51.064	21.014	1.00	62.83	C
ATOM	1000	NZ	LYS	C	27	13.750	50.060	20.828	1.00	64.49	C
ATOM	1001	C	LYS	C	27	7.299	52.556	20.750	1.00	52.44	C
ATOM	1002	O	LYS	C	27	7.334	53.710	21.165	1.00	54.11	C
ATOM	1003	N	LYS	C	28	6.877	52.239	19.538	1.00	53.88	C
ATOM	1004	CA	LYS	C	28	6.435	53.250	18.599	1.00	55.29	C
ATOM	1005	CB	LYS	C	28	6.169	52.582	17.249	1.00	57.59	C
ATOM	1006	CG	LYS	C	28	7.390	51.841	16.717	1.00	59.15	C
ATOM	1007	CD	LYS	C	28	7.041	50.830	15.635	1.00	62.19	C
ATOM	1008	CE	LYS	C	28	8.292	50.088	15.158	1.00	63.12	C
ATOM	1009	NZ	LYS	C	28	9.029	49.411	16.282	1.00	65.69	C
ATOM	1010	C	LYS	C	28	5.187	53.931	19.122	1.00	55.14	C
ATOM	1011	O	LYS	C	28	5.052	55.147	19.030	1.00	57.43	C

Figure 11Q

ATOM	1012	N	LEU	C	29	4.275	53.138	19.671	1.00	52.27	C
ATOM	1013	CA	LEU	C	29	3.025	53.649	20.214	1.00	51.04	C
ATOM	1014	CB	LEU	C	29	2.281	52.485	20.855	1.00	51.13	C
ATOM	1015	CG	LEU	C	29	0.776	52.493	21.051	1.00	50.66	C
ATOM	1016	CD1	LEU	C	29	0.051	52.868	19.755	1.00	51.59	C
ATOM	1017	CD2	LEU	C	29	0.389	51.100	21.491	1.00	50.29	C
ATOM	1018	C	LEU	C	29	3.347	54.739	21.245	1.00	50.83	C
ATOM	1019	O	LEU	C	29	2.739	55.805	21.269	1.00	53.58	C
ATOM	1020	N	LEU	C	30	4.327	54.457	22.089	1.00	50.52	C
ATOM	1021	CA	LEU	C	30	4.767	55.397	23.100	1.00	48.88	C
ATOM	1022	CB	LEU	C	30	5.813	54.730	23.997	1.00	48.03	C
ATOM	1023	CG	LEU	C	30	6.485	55.530	25.113	1.00	47.31	C
ATOM	1024	CD1	LEU	C	30	5.447	56.172	26.033	1.00	45.24	C
ATOM	1025	CD2	LEU	C	30	7.398	54.575	25.889	1.00	48.28	C
ATOM	1026	C	LEU	C	30	5.374	56.587	22.379	1.00	48.83	C
ATOM	1027	O	LEU	C	30	5.020	57.736	22.642	1.00	48.40	C
ATOM	1028	N	GLN	C	31	6.298	56.289	21.470	1.00	49.93	C
ATOM	1029	CA	GLN	C	31	6.983	57.304	20.670	1.00	52.00	C
ATOM	1030	CB	GLN	C	31	7.822	56.609	19.590	1.00	55.56	C
ATOM	1031	CG	GLN	C	31	8.628	57.513	18.645	1.00	61.26	C
ATOM	1032	CD	GLN	C	31	9.768	58.241	19.333	1.00	64.58	C
ATOM	1033	OE1	GLN	C	31	10.233	57.818	20.391	1.00	68.00	C
ATOM	1034	NE2	GLN	C	31	10.249	59.318	18.715	1.00	64.37	C
ATOM	1035	C	GLN	C	31	5.947	58.225	20.009	1.00	49.56	C
ATOM	1036	O	GLN	C	31	6.192	59.415	19.814	1.00	45.68	C
ATOM	1037	N	LEU	C	32	4.793	57.657	19.675	1.00	47.64	C
ATOM	1038	CA	LEU	C	32	3.723	58.401	19.034	1.00	48.95	C
ATOM	1039	CB	LEU	C	32	2.689	57.433	18.461	1.00	50.72	C
ATOM	1040	CG	LEU	C	32	1.602	57.935	17.502	1.00	51.93	C
ATOM	1041	CD1	LEU	C	32	2.209	58.293	16.154	1.00	50.26	C
ATOM	1042	CD2	LEU	C	32	0.554	56.840	17.313	1.00	51.55	C
ATOM	1043	C	LEU	C	32	3.070	59.295	20.077	1.00	49.32	C
ATOM	1044	O	LEU	C	32	3.040	60.519	19.929	1.00	50.01	C
ATOM	1045	N	THR	C	33	2.545	58.659	21.125	1.00	48.74	C
ATOM	1046	CA	THR	C	33	1.878	59.324	22.246	1.00	43.86	C
ATOM	1047	CB	THR	C	33	1.643	58.329	23.400	1.00	46.04	C
ATOM	1048	OG1	THR	C	33	0.707	57.332	22.977	1.00	47.18	C
ATOM	1049	CG2	THR	C	33	1.121	59.039	24.639	1.00	42.89	C
ATOM	1050	C	THR	C	33	2.683	60.494	22.771	1.00	41.04	C
ATOM	1051	O	THR	C	33	2.132	61.537	23.122	1.00	39.26	C
ATOM	1052	N	VAL	C	34	3.992	60.303	22.843	1.00	38.83	C
ATOM	1053	CA	VAL	C	34	4.886	61.346	23.301	1.00	36.90	C
ATOM	1054	CB	VAL	C	34	6.329	60.825	23.377	1.00	33.71	C
ATOM	1055	CG1	VAL	C	34	7.270	61.907	23.904	1.00	29.40	C
ATOM	1056	CG2	VAL	C	34	6.366	59.590	24.251	1.00	31.78	C
ATOM	1057	C	VAL	C	34	4.795	62.437	22.254	1.00	38.65	C
ATOM	1058	O	VAL	C	34	4.489	63.595	22.556	1.00	39.38	C
ATOM	1059	N	TRP	C	35	5.049	62.038	21.010	1.00	42.18	C
ATOM	1060	CA	TRP	C	35	5.002	62.937	19.868	1.00	40.00	C
ATOM	1061	CB	TRP	C	35	4.991	62.134	18.563	1.00	40.06	C
ATOM	1062	CG	TRP	C	35	4.848	63.020	17.399	1.00	36.56	C
ATOM	1063	CD2	TRP	C	35	3.696	63.161	16.561	1.00	36.91	C
ATOM	1064	CE2	TRP	C	35	3.968	64.212	15.673	1.00	41.20	C
ATOM	1065	CE3	TRP	C	35	2.457	62.505	16.503	1.00	40.31	C
ATOM	1066	CD1	TRP	C	35	5.748	63.944	16.974	1.00	35.30	C
ATOM	1067	NE1	TRP	C	35	5.228	64.673	15.945	1.00	39.45	C
ATOM	1068	CZ2	TRP	C	35	3.037	64.643	14.704	1.00	38.75	C
ATOM	1069	CZ3	TRP	C	35	1.528	62.934	15.541	1.00	39.54	C
ATOM	1070	CH2	TRP	C	35	1.827	63.984	14.651	1.00	41.30	C
ATOM	1071	C	TRP	C	35	3.764	63.833	19.901	1.00	39.80	C

Figure 11R

ATOM	1072	O	TRP	C	35	3.868	65.052	19.769	1.00	38.39	C
ATOM	1073	N	GLY	C	36	2.601	63.210	20.059	1.00	36.88	C
ATOM	1074	CA	GLY	C	36	1.356	63.957	20.103	1.00	38.94	C
ATOM	1075	C	GLY	C	36	1.315	64.973	21.226	1.00	38.45	C
ATOM	1076	O	GLY	C	36	0.931	66.114	21.001	1.00	37.76	C
ATOM	1077	N	ILE	C	37	1.700	64.557	22.435	1.00	40.86	C
ATOM	1078	CA	ILE	C	37	1.724	65.442	23.604	1.00	36.66	C
ATOM	1079	CB	ILE	C	37	2.352	64.755	24.857	1.00	36.83	C
ATOM	1080	CG2	ILE	C	37	2.489	65.766	26.005	1.00	27.32	C
ATOM	1081	CG1	ILE	C	37	1.512	63.548	25.275	1.00	37.14	C
ATOM	1082	CD1	ILE	C	37	2.066	62.794	26.501	1.00	35.85	C
ATOM	1083	C	ILE	C	37	2.587	66.655	23.284	1.00	38.37	C
ATOM	1084	O	ILE	C	37	2.187	67.788	23.529	1.00	37.68	C
ATOM	1085	N	LYS	C	38	3.775	66.399	22.740	1.00	40.28	C
ATOM	1086	CA	LYS	C	38	4.717	67.456	22.381	1.00	42.51	C
ATOM	1087	CB	LYS	C	38	5.927	66.868	21.639	1.00	46.36	C
ATOM	1088	CG	LYS	C	38	7.109	67.829	21.391	1.00	52.00	C
ATOM	1089	CD	LYS	C	38	8.125	67.162	20.433	1.00	56.10	C
ATOM	1090	CE	LYS	C	38	9.365	68.008	20.096	1.00	56.61	C
ATOM	1091	NZ	LYS	C	38	10.340	68.155	21.222	1.00	59.57	C
ATOM	1092	C	LYS	C	38	3.995	68.445	21.483	1.00	42.06	C
ATOM	1093	O	LYS	C	38	4.351	69.628	21.428	1.00	41.76	C
ATOM	1094	N	GLN	C	39	2.977	67.966	20.774	1.00	39.73	C
ATOM	1095	CA	GLN	C	39	2.232	68.862	19.908	1.00	40.05	C
ATOM	1096	CB	GLN	C	39	1.499	68.100	18.778	1.00	41.27	C
ATOM	1097	CG	GLN	C	39	2.385	67.148	17.928	1.00	42.44	C
ATOM	1098	CD	GLN	C	39	3.681	67.786	17.465	1.00	42.00	C
ATOM	1099	OE1	GLN	C	39	3.678	68.822	16.813	1.00	44.81	C
ATOM	1100	NE2	GLN	C	39	4.802	67.163	17.802	1.00	46.04	C
ATOM	1101	C	GLN	C	39	1.241	69.638	20.781	1.00	36.22	C
ATOM	1102	O	GLN	C	39	1.344	70.845	20.885	1.00	34.78	C
ATOM	1103	N	LEU	C	40	0.285	68.950	21.398	1.00	34.58	C
ATOM	1104	CA	LEU	C	40	-0.696	69.625	22.268	1.00	36.22	C
ATOM	1105	CB	LEU	C	40	-1.465	68.595	23.096	1.00	34.67	C
ATOM	1106	CG	LEU	C	40	-2.365	69.192	24.186	1.00	34.61	C
ATOM	1107	CD1	LEU	C	40	-3.392	70.142	23.510	1.00	34.28	C
ATOM	1108	CD2	LEU	C	40	-3.057	68.084	24.972	1.00	30.45	C
ATOM	1109	C	LEU	C	40	-0.029	70.630	23.226	1.00	36.51	C
ATOM	1110	O	LEU	C	40	-0.494	71.755	23.419	1.00	36.17	C
ATOM	1111	N	GLN	C	41	1.068	70.220	23.832	1.00	36.56	C
ATOM	1112	CA	GLN	C	41	1.764	71.106	24.751	1.00	38.77	C
ATOM	1113	CB	GLN	C	41	2.883	70.310	25.433	1.00	38.66	C
ATOM	1114	CG	GLN	C	41	3.606	70.994	26.582	1.00	46.41	C
ATOM	1115	CD	GLN	C	41	4.245	69.979	27.529	1.00	50.25	C
ATOM	1116	OE1	GLN	C	41	4.898	69.028	27.091	1.00	53.94	C
ATOM	1117	NE2	GLN	C	41	4.063	70.180	28.831	1.00	52.04	C
ATOM	1118	C	GLN	C	41	2.291	72.336	23.998	1.00	37.58	C
ATOM	1119	O	GLN	C	41	2.190	73.466	24.486	1.00	38.73	C
ATOM	1120	N	ALA	C	42	2.827	72.128	22.795	1.00	36.75	C
ATOM	1121	CA	ALA	C	42	3.365	73.249	22.014	1.00	36.93	C
ATOM	1122	CB	ALA	C	42	4.084	72.717	20.779	1.00	32.48	C
ATOM	1123	C	ALA	C	42	2.241	74.209	21.600	1.00	35.85	C
ATOM	1124	O	ALA	C	42	2.407	75.427	21.602	1.00	34.18	C
ATOM	1125	N	ARG	C	43	1.101	73.629	21.249	1.00	32.51	C
ATOM	1126	CA	ARG	C	43	-0.072	74.365	20.829	1.00	34.59	C
ATOM	1127	CB	ARG	C	43	-1.152	73.357	20.524	1.00	34.80	C
ATOM	1128	CG	ARG	C	43	-2.467	73.891	20.060	1.00	36.54	C
ATOM	1129	CD	ARG	C	43	-3.310	72.667	19.769	1.00	37.93	C
ATOM	1130	NE	ARG	C	43	-4.631	72.945	19.236	1.00	40.51	C
ATOM	1131	CZ	ARG	C	43	-5.481	71.985	18.901	1.00	42.74	C

Figure 11S

ATOM	1132	NH1	ARG	C	43	-5.127	70.717	19.051	1.00	41.40	C
ATOM	1133	NH2	ARG	C	43	-6.676	72.288	18.421	1.00	44.00	C
ATOM	1134	C	ARG	C	43	-0.568	75.347	21.883	1.00	37.96	C
ATOM	1135	O	ARG	C	43	-1.049	76.425	21.558	1.00	36.78	C
ATOM	1136	N	ILE	C	44	-0.434	74.971	23.151	1.00	41.66	C
ATOM	1137	CA	ILE	C	44	-0.901	75.799	24.250	1.00	43.04	C
ATOM	1138	CB	ILE	C	44	-1.403	74.891	25.390	1.00	45.88	C
ATOM	1139	CG2	ILE	C	44	-1.802	75.717	26.594	1.00	46.21	C
ATOM	1140	CG1	ILE	C	44	-2.572	74.041	24.876	1.00	46.16	C
ATOM	1141	CD1	ILE	C	44	-2.926	72.877	25.786	1.00	50.31	C
ATOM	1142	C	ILE	C	44	0.109	76.802	24.807	1.00	41.15	C
ATOM	1143	O	ILE	C	44	-0.235	77.961	25.047	1.00	40.03	C
ATOM	1144	N	LEU	C	45	1.345	76.350	25.005	1.00	40.33	C
ATOM	1145	CA	LEU	C	45	2.401	77.184	25.579	1.00	39.81	C
ATOM	1146	CB	LEU	C	45	3.357	76.322	26.422	1.00	40.22	C
ATOM	1147	CG	LEU	C	45	2.889	75.608	27.694	1.00	40.80	C
ATOM	1148	CD1	LEU	C	45	1.733	74.714	27.364	1.00	42.51	C
ATOM	1149	CD2	LEU	C	45	4.029	74.789	28.299	1.00	39.44	C
ATOM	1150	C	LEU	C	45	3.215	77.953	24.540	1.00	38.95	C
ATOM	1151	O	LEU	C	45	3.071	77.689	23.327	1.00	39.83	C
ATOM	1152	NT	LEU	C	45	4.014	78.810	24.964	1.00	39.47	C
ATOM	1153	OH2	TIP	W	2	8.280	62.369	27.138	1.00	38.82	W
ATOM	1154	OH2	TIP	W	3	28.782	24.001	17.582	1.00	78.47	W
ATOM	1155	OH2	TIP	W	4	0.492	62.209	33.896	1.00	50.43	W
ATOM	1156	OH2	TIP	W	5	6.020	70.609	23.199	1.00	45.29	W
ATOM	1157	OH2	TIP	W	6	1.993	78.695	31.896	1.00	37.25	W
ATOM	1158	OH2	TIP	W	7	20.294	18.975	19.485	1.00	49.56	W
ATOM	1159	OH2	TIP	W	8	18.592	15.442	35.405	1.00	34.86	W
ATOM	1160	OH2	TIP	W	9	-5.907	64.337	32.524	1.00	31.24	W
ATOM	1161	OH2	TIP	W	10	11.567	18.853	30.945	1.00	47.94	W
ATOM	1162	OH2	TIP	W	11	-9.321	65.456	23.794	1.00	46.60	W
ATOM	1163	OH2	TIP	W	12	-2.842	65.953	28.078	1.00	59.15	W
ATOM	1164	OH2	TIP	W	13	-1.409	77.305	18.859	1.00	37.51	W
ATOM	1165	OH2	TIP	W	14	-5.597	64.224	37.408	1.00	39.02	W
ATOM	1166	OH2	TIP	W	15	-5.079	75.908	18.460	1.00	48.65	W
ATOM	1167	OH2	TIP	W	16	12.444	58.431	21.920	1.00	62.97	W
ATOM	1168	OH2	TIP	W	17	-12.927	70.555	24.520	1.00	61.81	W
ATOM	1169	OH2	TIP	W	18	14.897	23.356	34.046	1.00	40.13	W
ATOM	1170	OH2	TIP	W	19	3.154	40.721	28.964	1.00	29.89	W
ATOM	1171	OH2	TIP	W	20	4.290	81.951	24.440	1.00	44.83	W
ATOM	1172	OH2	TIP	W	21	26.490	23.104	32.265	1.00	62.67	W
ATOM	1173	OH2	TIP	W	22	13.085	59.162	33.622	1.00	54.53	W
ATOM	1174	OH2	TIP	W	23	-0.166	45.626	35.200	1.00	56.34	W
ATOM	1175	OH2	TIP	W	24	-10.278	62.692	33.857	1.00	64.05	W
ATOM	1176	OH2	TIP	W	25	22.697	10.892	29.710	1.00	100.00	W
ATOM	1177	OH2	TIP	W	26	4.281	39.194	26.136	1.00	62.29	W
ATOM	1178	OH2	TIP	W	27	22.833	20.843	19.882	1.00	59.57	W
ATOM	1179	OH2	TIP	W	28	-10.030	74.838	23.517	1.00	53.18	W
ATOM	1180	OH2	TIP	W	29	1.246	80.456	24.973	1.00	36.18	W
ATOM	1181	OH2	TIP	W	30	-3.034	76.181	17.506	1.00	50.44	W
ATOM	1182	OH2	TIP	W	31	1.424	49.275	18.155	1.00	44.03	W
ATOM	1183	OH2	TIP	W	32	6.269	64.921	23.710	1.00	31.68	W
ATOM	1184	OH2	TIP	W	33	27.134	28.497	40.798	1.00	60.31	W
ATOM	1185	OH2	TIP	W	34	24.326	28.221	41.517	1.00	85.52	W
ATOM	1186	OH2	TIP	W	35	24.492	26.009	31.850	1.00	68.20	W
ATOM	1187	OH2	TIP	W	36	17.270	23.540	41.621	1.00	45.61	W
ATOM	1188	OH2	TIP	W	37	17.175	27.169	41.299	1.00	57.26	W
ATOM	1189	OH2	TIP	W	38	17.133	30.154	42.769	1.00	94.65	W
ATOM	1190	OH2	TIP	W	39	23.961	29.473	38.207	1.00	73.43	W
ATOM	1191	OH2	TIP	W	40	26.646	30.299	35.030	1.00	86.46	W

Figure 11T

ATOM	1192	OH2	TIP	W	41	21.799	33.921	37.475	1.00	98.23	W
ATOM	1193	OH2	TIP	W	42	12.296	24.508	37.800	1.00	73.10	W
ATOM	1194	OH2	TIP	W	43	10.910	28.524	40.599	1.00	65.23	W
ATOM	1195	OH2	TIP	W	44	8.726	30.065	36.214	1.00	62.46	W
ATOM	1196	OH2	TIP	W	45	20.748	34.061	34.804	1.00	62.12	W
ATOM	1197	OH2	TIP	W	46	7.462	29.159	29.170	1.00	88.23	W
ATOM	1198	OH2	TIP	W	47	7.466	31.280	33.124	1.00	56.10	W
ATOM	1199	OH2	TIP	W	48	6.666	26.619	36.241	1.00	52.76	W
ATOM	1200	OH2	TIP	W	49	3.823	27.148	35.557	1.00	92.76	W
ATOM	1201	OH2	TIP	W	50	7.608	28.183	32.367	1.00	83.54	W
ATOM	1202	OH2	TIP	W	51	10.064	35.767	38.975	1.00	68.12	W
ATOM	1203	OH2	TIP	W	52	14.649	36.973	38.236	1.00	73.09	W
ATOM	1204	OH2	TIP	W	53	16.799	36.406	39.778	1.00	48.69	W
ATOM	1205	OH2	TIP	W	54	15.456	39.954	39.598	1.00	48.97	W
ATOM	1206	OH2	TIP	W	55	8.442	41.891	37.753	1.00	57.63	W
ATOM	1207	OH2	TIP	W	56	9.926	44.040	39.986	1.00	80.20	W
ATOM	1208	OH2	TIP	W	57	3.713	35.630	32.034	1.00	65.94	W
ATOM	1209	OH2	TIP	W	58	4.004	32.569	30.481	1.00	98.02	W
ATOM	1210	OH2	TIP	W	59	13.514	45.594	36.374	1.00	45.92	W
ATOM	1211	OH2	TIP	W	60	12.274	44.358	32.693	1.00	69.72	W
ATOM	1212	OH2	TIP	W	61	-1.770	41.459	30.288	1.00	86.62	W
ATOM	1213	OH2	TIP	W	62	-0.747	39.619	34.003	1.00	85.57	W
ATOM	1214	OH2	TIP	W	63	2.370	42.056	36.997	1.00	63.26	W
ATOM	1215	OH2	TIP	W	64	7.646	47.813	26.559	1.00	86.77	W
ATOM	1216	OH2	TIP	W	65	-1.942	50.096	25.818	1.00	33.47	W
ATOM	1217	OH2	TIP	W	66	-0.455	48.262	24.057	1.00	48.49	W
ATOM	1218	OH2	TIP	W	67	-1.850	44.976	32.352	1.00	46.88	W
ATOM	1219	OH2	TIP	W	68	-4.779	47.469	30.587	1.00	53.38	W
ATOM	1220	OH2	TIP	W	69	-8.800	47.417	33.155	1.00	55.34	W
ATOM	1221	OH2	TIP	W	70	-7.762	51.374	35.608	1.00	72.46	W
ATOM	1222	OH2	TIP	W	71	5.493	50.307	35.418	1.00	63.93	W
ATOM	1223	OH2	TIP	W	72	-2.293	60.557	33.176	1.00	58.13	W
ATOM	1224	OH2	TIP	W	73	-3.891	59.956	22.859	1.00	42.99	W
ATOM	1225	OH2	TIP	W	74	-2.324	52.365	23.808	1.00	68.12	W
ATOM	1226	OH2	TIP	W	75	-4.610	53.603	23.534	1.00	99.86	W
ATOM	1227	OH2	TIP	W	76	-5.369	51.351	24.806	1.00	66.59	W
ATOM	1228	OH2	TIP	W	77	-9.158	53.927	27.711	1.00	59.38	W
ATOM	1229	OH2	TIP	W	78	-6.839	60.379	22.155	1.00	48.43	W
ATOM	1230	OH2	TIP	W	79	-7.811	55.209	31.835	1.00	63.25	W
ATOM	1231	OH2	TIP	W	80	-8.988	55.740	34.680	1.00	48.03	W
ATOM	1232	OH2	TIP	W	81	-14.358	62.793	31.478	1.00	77.34	W
ATOM	1233	OH2	TIP	W	82	-14.884	67.194	30.264	1.00	100.00	W
ATOM	1234	OH2	TIP	W	83	-13.964	62.903	27.850	1.00	61.59	W
ATOM	1235	OH2	TIP	W	84	-16.467	64.338	27.598	1.00	62.99	W
ATOM	1236	OH2	TIP	W	85	-14.165	71.419	31.235	1.00	58.55	W
ATOM	1237	OH2	TIP	W	86	-12.150	75.052	20.683	1.00	54.74	W
ATOM	1238	OH2	TIP	W	87	-15.348	66.527	23.972	1.00	86.65	W
ATOM	1239	OH2	TIP	W	88	23.657	18.784	16.110	1.00	46.11	W
ATOM	1240	OH2	TIP	W	89	21.774	13.448	17.383	1.00	55.62	W
ATOM	1241	OH2	TIP	W	90	28.955	20.801	18.398	1.00	47.29	W
ATOM	1242	OH2	TIP	W	91	19.043	22.428	18.931	1.00	70.31	W
ATOM	1243	OH2	TIP	W	92	32.348	21.741	32.055	1.00	80.85	W
ATOM	1244	OH2	TIP	W	93	31.544	26.386	31.293	1.00	80.53	W
ATOM	1245	OH2	TIP	W	94	30.484	31.504	24.099	1.00	51.19	W
ATOM	1246	OH2	TIP	W	95	28.981	30.812	18.458	1.00	98.45	W
ATOM	1247	OH2	TIP	W	96	25.233	35.680	28.569	1.00	53.47	W
ATOM	1248	OH2	TIP	W	97	25.740	37.432	31.266	1.00	96.40	W
ATOM	1249	OH2	TIP	W	98	18.343	27.853	17.008	1.00	87.39	W
ATOM	1250	OH2	TIP	W	99	26.162	40.002	24.887	1.00	63.29	W
ATOM	1251	OH2	TIP	W	100	18.896	37.649	32.149	1.00	75.85	W

Figure 11U

ATOM	1252	OH2	TIP	W	101	20.897	31.301	18.264	1.00	88.40	W
ATOM	1253	OH2	TIP	W	102	19.191	42.582	21.453	1.00	55.18	W
ATOM	1254	OH2	TIP	W	103	23.958	41.188	26.907	1.00	78.30	W
ATOM	1255	OH2	TIP	W	104	18.433	46.716	22.932	1.00	54.59	W
ATOM	1256	OH2	TIP	W	105	22.353	48.547	25.042	1.00	59.94	W
ATOM	1257	OH2	TIP	W	106	21.797	41.049	34.496	1.00	78.60	W
ATOM	1258	OH2	TIP	W	107	21.437	46.210	33.535	1.00	75.53	W
ATOM	1259	OH2	TIP	W	108	14.907	43.959	21.380	1.00	54.65	W
ATOM	1260	OH2	TIP	W	109	15.635	42.456	19.119	1.00	58.03	W
ATOM	1261	OH2	TIP	W	110	19.533	44.310	33.666	1.00	80.58	W
ATOM	1262	OH2	TIP	W	111	18.747	50.736	29.399	1.00	60.97	W
ATOM	1263	OH2	TIP	W	112	21.131	52.757	28.680	1.00	55.70	W
ATOM	1264	OH2	TIP	W	113	17.303	55.311	38.133	1.00	72.59	W
ATOM	1265	OH2	TIP	W	114	18.939	58.215	28.845	1.00	79.75	W
ATOM	1266	OH2	TIP	W	115	14.666	59.680	28.964	1.00	50.64	W
ATOM	1267	OH2	TIP	W	116	17.408	62.649	28.523	1.00	74.43	W
ATOM	1268	OH2	TIP	W	117	12.106	61.533	23.810	1.00	89.64	W
ATOM	1269	OH2	TIP	W	118	10.138	60.131	37.626	1.00	89.60	W
ATOM	1270	OH2	TIP	W	119	14.125	60.999	36.831	1.00	78.03	W
ATOM	1271	OH2	TIP	W	120	6.987	65.584	27.400	1.00	63.28	W
ATOM	1272	OH2	TIP	W	121	8.699	65.761	30.950	1.00	64.96	W
ATOM	1273	OH2	TIP	W	122	11.912	66.582	33.458	1.00	45.24	W
ATOM	1274	OH2	TIP	W	123	7.712	69.520	31.053	1.00	89.81	W
ATOM	1275	OH2	TIP	W	124	0.300	66.328	28.053	1.00	83.63	W
ATOM	1276	OH2	TIP	W	125	18.739	12.093	36.575	1.00	68.16	W
ATOM	1277	OH2	TIP	W	126	8.341	17.901	23.874	1.00	69.12	W
ATOM	1278	OH2	TIP	W	127	6.665	20.667	30.766	1.00	79.31	W
ATOM	1279	OH2	TIP	W	128	13.178	21.216	32.239	1.00	55.97	W
ATOM	1280	OH2	TIP	W	129	7.700	21.187	21.255	1.00	66.56	W
ATOM	1281	OH2	TIP	W	130	17.038	26.024	19.828	1.00	40.17	W
ATOM	1282	OH2	TIP	W	131	9.682	31.384	16.376	1.00	77.12	W
ATOM	1283	OH2	TIP	W	132	11.568	29.117	15.187	1.00	59.43	W
ATOM	1284	OH2	TIP	W	133	2.602	30.287	27.387	1.00	64.52	W
ATOM	1285	OH2	TIP	W	134	10.743	41.812	16.813	1.00	84.35	W
ATOM	1286	OH2	TIP	W	135	13.070	38.706	12.664	1.00	61.24	W
ATOM	1287	OH2	TIP	W	136	9.262	44.518	14.939	1.00	51.92	W
ATOM	1288	OH2	TIP	W	137	12.139	53.137	17.554	1.00	56.22	W
ATOM	1289	OH2	TIP	W	138	14.403	57.453	15.838	1.00	66.72	W
ATOM	1290	OH2	TIP	W	139	11.017	71.423	23.035	1.00	71.76	W
ATOM	1291	OH2	TIP	W	140	10.451	75.718	24.795	1.00	58.85	W
ATOM	1292	OH2	TIP	W	141	11.223	65.048	21.172	1.00	84.46	W
ATOM	1293	OH2	TIP	W	142	8.196	70.691	21.387	1.00	66.14	W
ATOM	1294	OH2	TIP	W	143	3.381	51.168	17.717	1.00	51.91	W
ATOM	1295	OH2	TIP	W	144	13.735	48.059	19.325	1.00	73.18	W
ATOM	1296	OH2	TIP	W	145	2.524	42.027	17.393	1.00	80.66	W
ATOM	1297	OH2	TIP	W	146	2.024	39.150	18.549	1.00	74.07	W
ATOM	1298	OH2	TIP	W	147	0.486	41.584	19.991	1.00	97.41	W
ATOM	1299	OH2	TIP	W	148	0.060	40.945	24.577	1.00	78.10	W
ATOM	1300	OH2	TIP	W	149	14.261	36.624	16.034	1.00	71.76	W
ATOM	1301	OH2	TIP	W	150	17.041	33.288	18.134	1.00	55.41	W
ATOM	1302	OH2	TIP	W	151	12.012	53.850	23.650	1.00	34.32	W
ATOM	1303	OH2	TIP	W	152	0.421	41.869	28.444	1.00	53.88	W
ATOM	1304	CL-1	CL	I	1	13.184	36.734	27.569	1.00	62.34	I
END											

Figure 11V



## INHIBITORS OF HIV MEMBRANE FUSION

## RELATED APPLICATIONS

This application is related to U.S. Provisional Application 60/043,280, entitled Core Structure of gp41 from the HIV Envelope Glycoprotein, by David C. Chan, Deborah Fass, Min Lu, James M. Berger and Peter S. Kim, filed Apr. 17, 1997 and U.S. application Ser. No. 09/062,241, entitled Core Structure of gp41 from the HIV Envelope Glycoprotein, by David C. Chan, Deborah Fass, Min Lu, James M. Berger and Peter S. Kim, filed Apr. 17, 1998. The present application claims the benefit of U.S. Provisional Application 60/094,676, entitled Inhibitors of HIV Membrane Fusion by David C. Chan, Debra M. Ehrigott and Peter S. Kim, filed Jul. 30, 1998; U.S. Provisional Application 60/100,265, entitled Inhibitors of HIV Membrane Fusion, by David C. Chan, Debra M. Ehrigott and Peter S. Kim, filed Sep. 14, 1998 and U.S. Provisional Application 60/101,058, entitled Inhibitors of HIV Membrane Fusion, by David C. Chan, Debra M. Ehrigott and Peter S. Kim, filed Sep. 18, 1998; and U.S. Provisional Application 60/132,295, entitled Inhibitors of HIV Membrane Fusion, by Debra M. Ehrigott, David C. Chan, Vladimir Malashkevich and Peter S. Kim, filed May 3, 1999. The entire teachings of these referenced applications are incorporated herein by reference.

## GOVERNMENT SUPPORT

The invention was supported, in whole or in part, by National Institutes of Health Grant Number P01 GM56552. The United States Government has certain rights in the invention.

## BACKGROUND OF THE INVENTION

Structural studies of proteins from human immunodeficiency virus type I (HIV-1) have been essential in the development of anti-retroviral drugs. Structure-based drug development has been most intense for reverse transcriptase inhibitors and protease inhibitors, the two classes of HIV-1 drugs in clinical use. It would also be useful to be able to carry out structure-based drug development against HIV entry.

## SUMMARY OF THE INVENTION

As described herein, the cavities on the surface of the N-helix coiled-coil of HIV envelope protein gp41 subunit (e.g., HIV-1 envelope protein gp41-subunit) are targets for drugs or other agents which, by binding the coiled-coil surface, particularly the cavities, inhibit HIV entry into cells. This is useful as the basis for identifying and designing drugs or agents which inhibit entry of HIV (e.g., HIV-1, HIV-2) into cells.

Results described herein show that the coiled-coil cavity (also referred to as the hydrophobic pocket) in the gp41 core is an attractive drug target and that molecules which bind the cavity interfere with (inhibit) HIV infectivity (HIV entry into cells). Applicants have shown, for the first time, that conserved residues projecting into the hydrophobic pocket clearly play a major role in the ability of C34 to inhibit HIV-1 infection. The importance of cavity contacts (between the N-helix coiled-coil cavity and residues of the C peptide region of gp41) to gp41 function is clear. Conversely, the importance of preventing such cavity contacts in inhibiting gp41 function and, thus, inhibiting HIV-1 entry into cells, is also clear. In addition, directing drugs against the hydrophobic pocket of the central-coiled coil of gp41 targets one

of the most highly conserved regions of the HIV-1 envelope proteins, which means that drugs which target the coiled-coil surface, and particularly its hydrophobic pocket, will have broad activity against diverse HIV isolates and that it will be difficult for drug-escape mutants to emerge.

A variety of methods, such as mirror-image phage display techniques (T. N. Schumacher, et al., *Science*, 271:1854 (1996)), combinatorial chemistry (A. Borchardt, S. D. Liberles, S. R. Biggar, G. R. Crabtree, S. L. Schreiber, *Chem. Biol.*, 4:961 (1997); J. C. Chabala, *Curr. Opin. Biotechnol.*, 6:632 (1995)), rational drug design and other drug screening and medicinal chemistry methods can be used to identify D-peptides, peptidomimetics and small molecules that bind the coiled-coil cavity with sufficient affinity to inhibit HIV-1 infection. The close correlation between N36/C34 stability and C34 potency, described herein, suggests that the effectiveness of such compounds will depend critically on the strength of their cavity-contacts. As described herein, candidate compounds can be tested for their ability to interfere with formation of a stable complex between C34 and N36 or their ability to disrupt binding of the two (disrupt the complex), thereby providing rapid, quantitative screens to identify and evaluate potential inhibitors of HIV-1 entry.

Alternatively, screening can be carried out to identify molecules or compounds which interfere with or disrupt binding of the N-helix coiled-coil cavity and a peptide which binds the cavity, thus providing methods of identifying molecules which are "pocket specific" binding agents or drugs. Molecules and compounds described herein (also referred to as drugs or agents) are useful to inactivate gp41 and, thus, prevent or reduce (inhibit) HIV-1 entry into cells. Without wishing to be bound by theory, it is reasonable to propose that these inhibitors bind to the pre-hairpin intermediate of gp41 and prevent its conversion to the trimeric hairpin structure of the gp41 core which corresponds to the fusion-active state of gp41. (Chan, D. C. and P. S. Kim, *Cell*, 93:681 (1998), See FIG. 1). Thus, the present methods are useful to identify drugs or agents which inhibit (totally or partially) formation of the fusion-active state of HIV-1 gp41 envelope protein. In the method, the ability of a candidate inhibitor (also referred to as a candidate drug), which can be any type of compound or molecule, such as a small molecule (e.g., a small organic molecule), a peptide (a D-peptide or an L-peptide), a peptidomimetic, a protein or an antibody, to bind the N-helix coiled-coil of gp41 and form a stable complex is assessed. Compounds or molecules which bind to the N-helix coiled-coil are further assessed for their ability to inhibit gp41 function (inhibit membrane fusion), such as through HIV-1 infection (viral entry) and syncytium assays, representative models of which are described and referenced herein. Those agents shown to inhibit gp41 function through such assays can be further assessed for their activity in additional in vitro assays and in appropriate animal models (e.g., Letvin, N. L., *Science*, 280, (5371): 1875-1880 (1998), Hirsch, V. M. and P. R. Johnson, *Virus Research*, 32 (2): 183-203 (1994); Reimann, K. A. et al., *J. Virol.*, 70 (10): 6922-6928 (1996)). Any suitable approach can be used to assess binding of candidate inhibitors to the N-helix coiled-coil and, as a result of the work described herein, to the N-helix coiled coil cavity. In one embodiment, the ability of a candidate inhibitor to bind the synthetic peptide N36 (described in Lu, M. et al., *J. Biomol. Struct. Dyn.* 15: 465 (1997), Chan, D. C. et al., *Cell*, 89, 263 (1997) and U.S. Provisional Application 60/043,280, entitled Core Structure of gp41 From the HIV Envelope Glycoprotein, by David C. Chan, Deborah Fass, Min Lu, James M. Berger and

Peter S. Kim, filed Apr. 17, 1997) is assessed. The stability of the resulting complexes is assessed using methods described herein.

In a particular embodiment of the method of identifying compounds or molecules (drugs or agents) which bind the N-helix coiled-coil cavity, a soluble model that presents the gp41 coiled-coil cavity is used. The six helix bundle of HIV gp41 consists of an internal trimeric coiled-coil, composed of three identical N-peptides, surrounded by three C-peptides which fit into a conserved hydrophobic groove on the outside of the trimeric coiled-coil. The C-terminal end of the trimeric coiled-coil contains a large cavity into which bulky hydrophobic groups from the C-peptide pack. This hydrophobic pocket is used as the target for anti-HIV drug discovery and/or design. Unfortunately, in the absence of the C-peptide, the N-peptide is aggregated and not 100% helical. Thus, simply using an N peptide from HIV-1 gp41, such as N36, N51 (Lu, M. et al., *Nature Struct. Biology*, 1995) or DP-107 (Wild et al., *PNAS* 89:10537-10541 (1992)) is unlikely to provide an effective model for the N-helix coiled-coil.

As described herein, Applicants have succeeded in producing a soluble, non-aggregating trimeric peptide model of the hydrophobic pocket of HIV gp41 and, thus, for the first time, have provided a model that properly presents this hydrophobic pocket or cavity (in a manner or configuration which forms a similar structure to the corresponding residues in the HIV gp41 structure). (The terms "pocket" and "cavity" are used interchangeably.) As described, a peptide (also referred to as a fusion protein) which includes a soluble, trimeric coiled coil portion and a portion from the N-peptide region of HIV gp41 that includes the amino acid residues which form the pocket or cavity of the N-helix coiled-coil of HIV gp41 (the pocket-comprising residues of the N-peptide) has been produced and shown to be such a soluble model, useful to identify molecules or compounds which inhibit HIV gp41 function and, thus, HIV entry into cells. The trimeric version of the coiled-coil in the peptide (also referred to as a fusion protein) can be the coiled-coil region of a protein which is not a protein of HIV (a non HIV protein, such as GCN4-pI<sub>Q</sub>I) or a protein of HIV origin (a protein derived from HIV or having the same or a similar amino acid sequence as an HIV protein). In a specific embodiment, the soluble, non-aggregating trimeric peptide model of the large cavity, referred to as IQN17, comprises a trimeric version of the coiled-coil region of GCN4, the yeast transcription activator, and a portion of the C-terminal end of the N peptide of gp41. IQN17 contains 29 residues of GCN4-pI<sub>Q</sub>I (formerly referred to as GCN4-pIQ in U.S. Provisional Application 60/101,058) (Eckert, D. M. et al. *J. Mol. Biol.*, 284:859-865 (1998)), including three mutations for increased solubility, and 17 residues of HIV; there is a one residue overlap between the two proteins, making the total length of the fusion protein 45 residues. The sequence of GCN4-pI<sub>Q</sub>I is: ac-RMKQIEDKIEEILSKQYHIEIARIKKLIGER (SEQ ID NO: 1). The HIV Sequence is: LLQLTVWGIKQLQARIL (SEQ ID NO:20). The sequence of IQN17 is: ac-RMKQIEDKIEEIESKQKKIENEIARIKKLLQLTVWGIKQLQARIL-am (SEQ ID No:2). The HIV portion is underlined in SEQ ID No: 2; ac- represents an N-terminal acetyl group and -am represents a C-terminal amide. The sequence of the soluble, trimeric version of the coiled-coil region of GCN4 (referred to as a soluble, trimeric coiled coil of GCN4) in IQN17 is: RMKQIEDKIEEIESKQKKIENEIARIKK (SEQ ID No: 25). The superhelix parameters such as rise and pitch

(Harbury, P. B. et al., *Nature* 371:80-83 (1994); Harbury et al., *PNAS* 92:8408-8412 (1995)) of the GCN4-pI<sub>Q</sub>I coiled coil are nearly identical to the HIV gp41 N-helix coiled coil. Therefore, the resulting fusion protein molecule (IQN17) is predicted to form a long trimeric coiled coil, which presents the N-peptide hydrophobic cavity at the C terminus. IQN17 is fully helical, as determined by circular dichroism, with a molar ellipticity at 222 nm of -36,000 deg cm<sup>2</sup> dmol<sup>-1</sup>. As determined by sedimentation equilibrium, IQN17 is close to a discrete trimeric species with a ratio of observed molecular weight to calculated molecular weight ranging from 3.00 to 3.16 times the monomer molecular weight at a concentration of 20 μM. As determined by X-ray crystallography, IQN17 presents the N-peptide hydrophobic pocket in a manner that is nearly identical to the pocket in the HIV gp41 N-helix coiled coil.

The IQN17 molecule (in the natural L-handedness or enantiomeric D-handedness) can be used in screens, including high-throughput drug screens, to identify molecules that bind to the coiled-coil pocket. The IQN17 molecule, in the D-handedness, has been used as a target in mirror image phage display (Schumacher et al., *Science*, 271: 1854, 1996) to identify small molecules (D-peptides) which bind to the hydrophobic pocket of gp41 (in the natural L-handedness) and inhibit HIV-membrane fusion. The desired target (the N-helix of HIV gp41 which includes the hydrophobic pocket) is the exact mirror image of the naturally-occurring target. It is used to screen a library or collection of compounds or molecules which are to be assessed for their ability to bind the mirror image of the naturally-occurring coiled-coil pocket. The mirror image of a compound or molecule found to bind the mirror image of the naturally-occurring gp41 pocket, will bind the gp41 pocket in the natural handedness. The library or collection screened can be of any type, such as a phage display library, peptide library, DNA library, RNA library, combinatorial library, collection of chemical agents or drugs, cell lysate, cell culture medium or supernatant containing products produced by cells. In the case of a phage display library, the D-target is used to screen phage coat proteins. Specific phage clones that bind to the target are identified and the mirror images of the expressed proteins are chemically synthesized with D-amino acids. By using IQN17 in mirror-image phage display, D-peptides that bind to the gp41 hydrophobic pocket have been identified. Further assessment has been carried out, as described, to demonstrate the ability of D-peptides to inhibit HIV gp41 function. D-peptides which bind the gp41 hydrophobic pocket and inhibit HIV infectivity have been identified. D-peptides which bind the hydrophobic pocket also will serve as lead molecules for drug development and/or reagents for drug discovery (where the drugs bind to the coiled-coil pocket and inhibit HIV infectivity). The IQN17 molecule, in the natural L-handedness, can be used in screens, including high-throughput screens, to identify molecules that bind to the coiled-coil pocket. IQN17 can be used to screen a collection or library of compounds or molecules which are to be assessed for their ability to bind the hydrophobic pocket. The library or collection screened can be of any type, such as a phage display library, RNA library, DNA library, peptide library, combinatorial library, collection of chemical agents or drugs, cell lysate, cell culture medium or supernatant containing products produced by cells. Compounds or molecules which bind the hydrophobic pocket also will serve as lead molecules for drug development and/or reagents for drug discovery.

Fusion proteins which are variants of IQN17 can be produced and used to screen for drugs which bind the gp41

N-helix coiled-coil pocket. Any of a wide variety of variations can be made in the GCN4-pI component of IQN17 and used in the method, provided that these changes do not alter the trimeric state of the coiled-coil. For example, the amino acid composition of the GCN4 component can be changed by the addition, substitution, modification and/or deletion of one or more amino acid residues, provided that the trimeric state of the coiled-coil is maintained. For example, the Asp residue in IQN17 (at a "f-position" of the coiled coil) can be replaced by any of the naturally-occurring amino acids. (O'Neil and DeGrado, *Science* 250:646 (1990)). Alternatively, this component of the fusion protein can be a trimeric version of the coiled-coil region of another protein, such as that from Moloney Murine Leukemia Virus (Fass, D. et al. *Nature Struct. Biology*, 3:465 (1996)), GCN4-pII (Harbury et al., *Nature*, 317:80, 1994) or the ABC heterotrimer (Nautiyal and Alber, *Protein Science* 8:84 (1999)).

Changes can also be made in the amino acid composition of the fusion protein component which is the C-terminal portion of the HIV gp41 N peptide to produce IQN17 variants. The C-terminal portion can be changed by the addition, substitution, modification and/or deletion of one or more amino acid residues. The amino acid composition of either or both components of the fusion protein can be altered, and there is no limit to the number or types of amino acid residue changes possible, provided that the trimeric state of the coiled-coil and the hydrophobic pocket of the N peptide of HIV gp41 are maintained. IQN17, IQN17 variants or any soluble model of the large cavity can be used to screen for drugs which bind the N-helix coiled-coil, especially the pocket, or for lead drug candidates or candidates for use in vaccine preparations, to be further screened using methods known to those of skill in the art, such as in a high throughput format.

Results described herein are useful to screen for inhibitors of HIV gp41 which are variants of C34 as described below. Once a variant of C34, such as a C34 variant which stably binds N36, has been identified, it can be used and further assessed as obtained or it can be modified (e.g., by altering, adding, deleting or substituting at least one amino acid residue or adding a non-amino acid substituent), if desired or needed (e.g., to enhance stability, solubility, bioavailability). Alternatively, a C34 variant can be assessed to determine if a shorter component (region of fewer amino acid residues) also is active as an inhibitor. As discussed herein, the three C34 residues Trp<sup>62a</sup>, Trp<sup>631</sup> and Ile<sup>635</sup> that pack into the deep, conserved pocket in the N36 trimer are critical for inhibitory activity. The observation that C34 variants that have a higher affinity for the N36 coiled-coil have more potent inhibitory activity against HIV infection forms the basis for screens to identify and evaluate potential inhibitors. For example, using the "split-synthesis" technique (Chen, C. L., et al. *Methods Enzymol.* 267:211-219 (1996); Lam, K. S. et al., *Nature*, 354: 82-84, (1991)) of combinatorial peptide chemistry, a library of C34 variants is synthesized in which the three critical hydrophobic residues are randomly replaced by chemical substitutions of varying hydrophobic character. This synthesis technique results in the generation of a vast library of beads, each containing many copies of a single variant C34 peptide (i.e., a "one-bead, one-compound" type of library). To identify C34 variants which stably bind the N-helix coiled-coil, a labeled version of N36 (or a modified N-peptide) is mixed with the peptide beads under conditions (e.g., elevated temperature) that restrict binding to only those C34 variants with the highest affinity. Binding is measured by detection of the label on the N-helix

peptide, using known methods. Simple modifications of the split-synthesis technique allow ready identification of the selected peptide sequence by mass spectroscopy (Youngquist, R. S. et al., *J. Amer. Chem. Soc.* 117, 3900-3906 (1995)). The C34 variants selected, particularly those with the highest binding affinities for N36, are tested in syncytium and infection assays for gp41 inhibitory activity. Truncated versions of these C34 variants, containing only the cavity-binding region, can also be tested for inhibitory activity. Alternatively, a library of other peptides to be assessed can be synthesized to generate a library of beads, each containing (having bound thereto) a peptide to be assessed. This library is analyzed as described above for the C34 variants and resulting hits (members with appropriate binding affinities for N36) are further analyzed for gp41 inhibitory activity. As a second example, the N36 peptide or the soluble variants described earlier, such as IQN17, GCN4-N-helix peptide can be used as a target for phage display or mirror-image phage display techniques to identify peptides that bind to the cavity.

IQN17 can also be used to raise antibodies (monoclonal and/or polyclonal) that bind to the coiled-coil cavity. IQN17 can further be used, either alone or in combination with other materials, in a vaccine, which will elicit the production of antibodies that bind to the coiled-coil in the individual to whom it is administered (the vaccinee), and thereby offer protection against infection and/or disease.

Peptides, both D-peptides and L-peptides, which fit into a deep hydrophobic pocket in the trimeric N-helix coiled-coil of HIV-1 envelope glycoprotein gp41 are also the subject of this invention. The D-peptides are the first molecules that have been shown to bind exclusively to the gp41 hydrophobic pocket. The observation that these D-peptides inhibit gp41-mediated membrane fusion processes (syncytia formation and viral infection) provides the first direct demonstration that HIV-1 infection can be inhibited by molecules that bind specifically to pocket. The validation of the gp41 hydrophobic pocket as a drug target sets the stage for the development of a new class of orally bioavailable anti-HIV drugs, that work by inhibiting viral entry into cells. Such drugs would be a useful addition to the current regimen used to treat HIV-1 infection with combination therapies. D-peptides, such as the D-peptides described herein, portions, modification and variants thereof and larger molecules (e.g., polypeptides) which comprise all or a portion of a D-peptide described herein, are useful to inhibit HIV membrane fusion and, thus, HIV entry into cells. D-peptides, corresponding to the D-amino acid version of phage sequences identified as described herein, are inhibitors of HIV-1 infection and syncytia formation. The C-terminal residues in these D-peptide inhibitors have the sequence pattern: CXXXXXEWXWLCAA-am (SEQ ID NO: 69). (In the phage-display library, the positions corresponding to the C residues were encoded as either C or S, the positions corresponding to the AA residues were encoded as such and the other 10 positions (indicated by X) were randomly encoded. The -am represents a C-terminal amide, added as part of the peptide synthesis procedure.) The N-terminal residues in the D-peptide inhibitors are, for example, ac-GA, ac-KKGA (SEQ ID NO: 70), or ac-KKKKGA (SEQ ID NO: 71). The ac- represents an N-terminal acetyl group added as part of the peptide synthesis procedure. The C-terminal amide and the N-terminal acetyl group are optional components of D-peptides of this invention. Other N-terminal residues can be included, in place of or in addition to those in the previous sentence, as desired (e.g., to increase solubility). For example, D-peptides of the following sequences are also the subject of this invention:

ac-XXCXXXXXEWXWLCXX-am (SEQ ID NO: 28);  
 ac-KKXXCXXXXXEWXWLCXX-am (SEQ ID NO: 29);  
 ac-KKKKXXCXXXXXEWXWLCXX-am (SEQ ID NO: 30);  
 ac-XXCXXXXXEWXWLCXXX-am (SEQ ID NO: 31);  
 ac-KKXXCXXXXXEWXWLCXXX-am (SEQ ID NO: 32); and  
 ac-KKKKXXCXXXXXEWXWLCXXX-am (SEQ ID NO: 33).

The amino acid residues are represented by the single letter convention and X represents any amino acid residue (naturally occurring or non-naturally occurring) or other moiety, such as a modified amino acid residue.

Further, the ten amino acid residue "core" (the 10-mer which is flanked at each end by a cysteine residue) of the 12 amino acid residue peptide, as well as portions, modifications and variants of the 10-mers are also useful to inhibit membrane fusion and entry of HIV into cells. Variants, portions and modifications of these peptides are also useful as inhibitors. As described further herein, D-peptides which comprise a consensus sequence (e.g., WXWL (SEQ ID NO: 23), EWXWL (SEQ ID NO: 24), CXXXXXEWXWLC (SEQ ID NO: 63) or a portion thereof) have been shown to bind the N-helix coiled-coil and are useful to inhibit membrane fusion and entry of HIV into cells. The enantiomeric peptides (D-peptides) do not serve as efficient substrates for enzymes, such as proteases and, therefore, are more resistant to proteolytic degradation than are L-peptides; they are also less immunogenic than are L-peptides.

Specific embodiments of D-peptides of the present invention are:

- (a) CDLKAKEFWWLC (SEQ ID NO: 3);
- (b) CEARHREAWWLC (SEQ ID NO: 4);
- (c) CELLGWEAWWLC (SEQ ID NO: 5);
- (d) CLLRAPEWGWLC (SEQ ID NO: 6);
- (e) CSRSQPEWEWLC (SEQ ID NO: 7);
- (f) CGLGQEEFWWLC (SEQ ID NO: 8);
- (g) CMRGWEWSWLC (SEQ ID NO: 9);
- (h) CPPLNKEAWWLC (SEQ ID NO: 10);
- (i) CVLKAKEFWWLC (SEQ ID NO: 11);
- (j) KKGACGLGQEEFWWLC (SEQ ID NO: 15);
- (k) KKGACELLGWEAWWLC (SEQ ID NO: 16);
- (l) KKKKGACELLGWEAWWLC (SEQ ID NO: 17);
- (m) KKGACMRGEWEWSWLC (SEQ ID NO: 18);
- (n) KKGACPLNKEAWWLC (SEQ ID NO: 19);
- (o) a D-peptide comprising WXWL (SEQ ID NO: 23);
- (p) a D-peptide comprising EWXWL (SEQ ID NO: 24);
- (q) a D-peptide comprising CXXXXXEWXWL (SEQ ID NO: 12)
- (r) ac-GACEARHREAWWLC (SEQ ID NO: 34);
- (r) ac-KKGACEARHREAWWLC (SEQ ID NO: 38);
- (t) ac-KKKKGACEARHREAWWLC (SEQ ID NO: 43);
- (u) ac-GACGLGQEEFWWLC (SEQ ID NO: 44);
- (v) ac-KKGACGLGQEEFWWLC (SEQ ID NO: 64);
- (w) ac-KKKKGACGLGQEEFWWLC (SEQ ID NO: 45)
- (x) ac-GACDLKAKEFWWLC (SEQ ID NO: 35);
- (y) ac-KKGACDLKAKEFWWLC (SEQ ID NO: 39);

- (z) ac-KKKKGACDLKAKEFWWLC (SEQ ID NO: 46);
- (a') ac-GACELLGWEAWWLC (SEQ ID NO: 47);
- (b') ac-KKGACELLGWEAWWLC (SEQ ID NO: 65);
- (c') ac-KKKKGACELLGWEAWWLC (SEQ ID NO: 66);
- (d') ac-GACSRQPEWEWLC (SEQ ID NO: 36);
- (e') ac-KKGACSRQPEWEWLC (SEQ ID NO: 40);
- (f) ac-KKKKGACSRQPEWEWLC (SEQ ID NO: 48);
- (g') ac-GACLLRAPEWGWLC (SEQ ID NO: 37);
- (h') ac-KKGACLLRAPEWGWLC (SEQ ID NO: 41);
- (i') ac-KKKKGACLLRAPEWGWLC (SEQ ID NO: 49);
- (j') ac-GACMRGEWEWSWLC (SEQ ID NO: 50);
- (k') ac-KKGACMRGEWEWSWLC (SEQ ID NO: 67);
- (l') ac-KKKKGACMRGEWEWSWLC (SEQ ID NO: 51);
- (m') ac-GACPLNKEAWWLC (SEQ ID NO: 52);
- (n') ac-KKGACPLNKEAWWLC (SEQ ID NO: 68);
- (o') ac-KKKKGACPLNKEAWWLC (SEQ ID NO: 53);
- (p') ac-GACXXXXXEWXWLC (SEQ ID NO: 54);
- (q') ac-KKGACXXXXXEWXWLC (SEQ ID NO: 55);
- (r') ac-KKKKGACXXXXXEWXWLC (SEQ ID NO: 56);
- (s') ac-XXCXXXXXEWXWLC (SEQ ID NO: 57);
- (t') ac-KKXXCXXXXXEWXWLC (SEQ ID NO: 58);
- (u') ac-KKKKXXCXXXXXEWXWLC (SEQ ID NO: 59);
- (v') ac-XXCXXXXXEWXWLC (SEQ ID NO: 60);
- (w') ac-KKXXCXXXXXEWXWLC (SEQ ID NO: 61);
- (x') ac-KKKKXXCXXXXXEWXWLC (SEQ ID NO: 62); and
- (y') a variant of a sequence of (a) through (x'), wherein the variant binds the N-helix coiled-coil cavity of HIV gp41, wherein ac- at the C-terminus and -am at the N-terminus are optional.

D-peptides described herein, which are ligands shown to bind the N-helix pocket, are also useful in drug screens to identify compounds or molecules (e.g., from chemical libraries, recombinantly produced products, naturally-occurring substances, culture media or supernatants) which bind the N-helix pocket and thus, are also inhibitors of HIV. For example, a competitive assay can be carried out by combining a D-peptide which binds the N-helix cavity (e.g., a D-peptide described herein); IQN17 (e.g., in the natural L-handedness), or another fusion protein which is a soluble model that presents the N-helix cavity; and a candidate

inhibitor (a compound or molecule to be assessed for its ability to bind the N-helix cavity). For example, D10pep5 or D10pep1, IQN17, and a candidate inhibitor (candidate drug) can be combined using buffer conditions and peptide concentrations appropriate for binding of D10pep5 or D10pep1 to IQN17. The extent to which binding of the D-peptide occurs is determined and compared to the extent to which binding occurs under the same conditions, but in the absence of a compound or molecule (referred to as a candidate drug or candidate inhibitor) to be assessed for its ability to bind the N-helix coiled-coil cavity of HIV gp41 envelope protein (in a control). If binding of D10pep5 or D10pep1 occurs to a lesser extent in the presence of the candidate inhibitor (test sample) than in its absence (control sample), the candidate inhibitor is a ligand which binds the N-helix coiled-coil cavity and, thus, is an inhibitor. Inhibitors identified in this manner can be further assessed for their activity in viral infectivity assays and syncytia formation assays, such as those described herein. Those inhibitors which show activity in such assays can be further assessed in an appropriate animal model or in humans.

Any method by which binding of the D-peptide, known to bind the N-helix cavity, can be detected can be used to assess whether the candidate inhibitor interferes with binding. For example, the D-peptide can be detectably labeled and the extent to which the label appears on the N-helix cavity (as a result of binding of the D-peptide) detected, in the presence and in the absence of the candidate inhibitor. If less label appears on the N-helix cavity of IQN17 (or other appropriate fusion protein) in the presence of the candidate inhibitor (in the test sample) than in its absence (in the control sample), then the candidate inhibitor is a ligand which binds the N-helix cavity (and interferes with binding of the D-peptide). Alternatively, the D-peptide (e.g., D10pep5 or D10pep1) and IQN17 can be labeled with a fluorophore (e.g., with EDANS; 5-(2'-aminoethyl) aminonaphthalene-1-sulfonic acid) with an appropriate quencher that quenches the fluorescent signal of the fluorophore when it is in close proximity (e.g., DABCYL; 4-(4'-dimethylaminophenylazo)benzoic acid). If the candidate inhibitor binds the N-helix cavity of IQN17, fluorescence is observed, since, as a result of binding of the candidate inhibitor, the D-peptide is not brought into sufficiently close proximity to the quencher to permit it to quench the reporter signal. Alternatively, the fluorescent reporter molecule can be on the IQN17 and an appropriate quencher on the D-peptide. In either case, the position of the reporter or quencher on IQN17 must be such that when the D-peptide binds the N-helix cavity, the reporter and quencher moieties are in sufficiently close proximity to each other that quenching occurs (Tyagi, S., et al., *Nature Biotechnology* 16:49 (1998)).

Also the subject of this invention are drugs (compounds and molecules) which bind the N-helix coiled-coil pocket of HIV gp41 and inhibit (partially or totally) HIV entry into cells. In one embodiment, these drugs can be identified as described herein or by other methods. Drugs which bind the N-helix coiled-coil pocket of HIV gp41 are useful as therapeutic agents (to prevent HIV entry into cells or reduce the extent to which it occurs), as research tools (e.g., to study the mechanism of HIV gp41 function) and to assess the rate of viral clearance by an individual (e.g., in an animal model or an infected human).

Also the subject of this invention are compositions, useful in methods of interfering with entry of HIV into a mucosal cell; these compositions comprise an appropriate carrier or base and at least one component selected from the group consisting of:

- (a) C34 peptide;
- (b) DP178;
- (c) T649;
- (d) T1249;
- (e) a derivative of (a)-(d);
- (f) a D-peptide which binds to the hydrophobic pocket of HIV gp41;
- (g) a derivative of (f);
- (h) a combination of two or more of (a)-(g); and
- (i) a molecule that inhibits HIV infectivity by binding to the N-helix coiled coil.

The compositions can comprise one such component or two or more components.

A further subject of this invention are compositions (e.g., proteins or proteinaceous materials) that can be used to elicit an immune response (e.g., antibody production) that will protect (partially or totally) against HIV infection and/or disease. Such compositions are useful as protective agents (e.g., vaccines) and to obtain antibodies (monoclonal and/or polyclonal) that are useful as research tools, diagnostic tools, drug screening reagents, and to assess viral dynamics (rates of production and clearance of virus) in animal models or infected humans.

Also the subject of this invention is a list of atomic coordinates for the X-ray crystal structure of the complex between IQN17 and D10pep1. Also the subject of this invention is a list of coordinates for the X-ray crystal structure of IQN17. These coordinates can be used (e.g., as an electronic file for computer graphics programs) to create a model of the complex which indicates how D10pep1 binds to the N-helix coiled-coil cavity and models of the N-helix coiled-coil cavity. Such models can be used, in methods known to those of skill in the art such as in computer graphics modeling, to build new models to evaluate the likelihood of binding to the N-helix coiled-coil cavity by other peptides, peptidomimetics, small molecules, drugs or other compounds. Such models can also be used to build new models for the structures of molecules (peptides, peptidomimetics, small organic molecules, drugs or other compounds) that bind the N-helix coiled-coil cavity (e.g., H. Kubinyi, *Curr. Op. Drug Discov. Develop.*, 1:16 (1998); P. L. Wood, *ibid*, 1:34 (1998); J. R. Morphy, *ibid*, 1:59 (1998)). These models and the corresponding lists of atomic coordinates can be used to identify, evaluate, discover and design more effective and/or new D-peptides, L-peptides, peptidomimetics, other small molecules or drugs that inhibit HIV infectivity, using methods known to those of skill in the art. A further subject of this invention is a method of producing or identifying a drug which fits (packs into, binds) the N-helix coiled-coil pocket of HIV gp41 through the use of atomic coordinates of a crystal, such as a crystal of a soluble, trimeric peptide model of the HIV gp41 hydrophobic pocket described herein (e.g., IQN17 or a variant thereof), a crystal of such a model in complex with a D-peptide (e.g., IQN17 or a variant thereof in complex with a D-peptide described herein, such as D10pep1) or a crystal of the N-peptide region of HIV gp41 comprising the amino acid residues which comprise the pocket of the N-helix coiled-coil of HIV gp41. The method comprises obtaining a crystal of the soluble model, such as the empty soluble model (not in complex with a D-peptide), obtaining the atomic coordinates of the crystal (e.g., of the crystal of the empty soluble model, such as IQN17); using the atomic coordinates obtained to define the N-helix coiled-coil pocket of HIV gp41; identifying a molecule or compound which fits the N-helix coiled-coil pocket and obtaining the molecule or

compound; contacting the molecule or compound with the N-helix coiled-coil pocket (e.g., by contacting it with a polypeptide which comprises the pocket (e.g., IQN17 or a variant thereof or the N-peptide) to assess (determine) the ability of the molecule or compound to fit the pocket of HIV gp41, wherein the molecule or compound fits the pocket, it is a drug which fits the N-helix coiled-coil pocket, whereby a drug which fits the pocket is produced. The atomic coordinates of the crystal can be obtained by X-ray diffraction studies or form a computer file or Protein Data Base (PDB), such as the PDB presented herein for IQN17 (FIGS. 11A-11V).

Similarly, the method can be carried out using a crystal of a soluble trimeric model in complex with a D-peptide (e.g., a D-peptide described herein, such as D10pep1) or a crystal of the N-peptide region of HIV gp41 which comprises the pocket of the N-helix coiled coil.

Drugs produced in this manner can be further assessed to conform their ability to fit into the pocket (e.g., by NMR) and can be assessed for their ability to inhibit HIV entry into cells (e.g., by a syncytia assay or infectivity assay).

The teachings and entire contents of all documents cited herein are expressly incorporated by reference into this application.

#### BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic of HIV-1 gp41 showing the N36 (SGIVQQNNLLRAIEQQHLLQLTVWGIKQLQARIL) (SEQ ID NO: 13) and C34 (WMEWDREINNYTSLIHSLEESQNQQEKNEQELL) (SEQ ID NO: 14) peptides located within two regions containing 4,3 hydrophobic heptad repeats (labeled heptad repeat 1 and heptad repeat 2, also referred to as N-peptide region and C-peptide region, respectively). The underlined residues in C34 were mutated in this study. Three of these residues (W, W and I) project into the N36 cavity, whereas two of these residues (M and R) do not. FP, fusion peptide; S-S, disulfide bond; TM, transmembrane region; INTRA, intraviral region.

FIG. 2 is a graph showing the correlation of C34 inhibitory potency with N36/C34 stability. C34 peptide variants containing substitutions at position Trp<sup>631</sup> were tested for inhibition of viral entry (filled circles) and cell-cell fusion (open circles). IC<sub>50</sub> values are plotted on a logarithmic scale against the *T<sub>m</sub>* (melting temperature) of the corresponding N36/C34 complex. The identities and chemical structures of the substitutions are drawn under the corresponding data points. In order of increasing hydrophobic bulk, the substitutions were: glycine (Gly), alanine (Ala), L- $\alpha$ -aminobutyric acid (Abu), valine (Val), leucine (Leu), phenylalanine (Phe), the wildtype residue tryptophan (Trp), and L- $\beta$ -(1-naphthyl) alanine (Nal). Error bars indicate the standard error from triplicate experiments.

FIG. 3 shows the amino acid sequences of D-peptides (SEQ ID NOS: 34, 38, 64, 35, 65, 66, 36, 40, 41, 67 and 68) and the consensus sequence (SEQ ID NO.: 12). As represented, each peptide is flanked by GA on the N-terminus and AA on the C-terminus, and comprises a blocking group at the N-terminus: (Acetyl-GA-C-10mer-C-AA-CONH<sub>2</sub>; this can also be represented as ac-GA-C-10mer-C-AA-am). The single letter conventions which are used to represent amino acid residues are as follows: G=glycine; A=alanine; C=cysteine; D=aspartic acid; L=leucine; K=lysine; E=glutamic acid; W=tryptophan; F=phenylalanine; R=arginine; H=histidine; S=serine; and Q=glutamine.

FIG. 4 is a schematic representation of mirror-image phage display with the D-IQN17 target, in which: (1) rounds

of phage selection are carried out to identify binders to D-IQN17; (2) individual clones are sequenced; (3) binding specificity is assessed by determining whether the phage bind to the gp41 region of D-IQN17; (4) D-peptides of those phage sequences which bind are produced; and (5) the anti-HIV activity of the D-peptides is assayed.

FIGS. 5A and 5B show the crystal structure of IQN17 bound to D10pep1. IQN17 is shown to be a continuous three-stranded coil, and binding of the conserved amino acid residues of D10pep1 is shown to be to the hydrophobic pocket of IQN17, formed by the 17 residues derived from HIV gp41. FIG. 5A shows IQN17, consisting of GCN4-pI<sub>Q</sub>I residues fused to HIV-1 gp41 residues and the binding of D10pep1 to the hydrophobic pocket of IQN17 (area within box). The D-peptide which binds to the pocket is represented by the branched extensions (i.e., stick representation). FIG. 5B is an enlargement of the area within the box and shows the conserved residues that pack into the pocket (Trp, Trp Leu) as well as a glutamic acid (Glu).

FIGS. 6A and 6B show results of syncytia assays, using the D-peptides described herein. FIG. 6A is a graphic representation of results of syncytia assays. FIG. 6B represents IC<sub>50</sub> data for D-peptides, with results from one or more experiments.

FIGS. 7A-7N are the PDB file which lists the atomic coordinates for the crystal structure of D10pep1 bound to IQN17, in which residues 0-28 of the A chain are derived from the GCN4-pI<sub>Q</sub>I sequence (with three mutations), residues 29-45 of the A chain are derived from the HIV gp41 sequence, residues 0-16 of the D chain represent the D-peptide, ordered water molecules are represented as W, and a bound chloride ion as chain I. Residue 0 represents the acetyl group. The PDB file represents a monomer; the trimer is formed by crystallographic symmetry.

FIGS. 8A and 8B show results of assessment of inhibition of HIV-1 membrane fusion by a D-peptide. FIG. 8A shows results of syncytia assay with no D-peptide. FIG. 8B shows results of syncytia assay with D-peptide.

FIGS. 9A-9C show results of <sup>1</sup>H NMR experiments characterizing the aromatic residues of IQN17/D-peptide complexes. FIG. 9A shows 1D-NMR spectra of D10pep1a (top), IQN17 (middle) and a 1:1 complex of D10pep1a and IQN17 (bottom). The x-axis is the same as for (C) below. Upfield peaks assigned to the four scalar-coupled aromatic ring protons of Trp-571 are indicated. The unmarked upfield peak of the bottom trace corresponds to an unassigned H $\alpha$  resonance. FIG. 9B shows 1D spectra of 1:1 complexes between IQN17 and each D-peptide (as labeled). The same four protons are indicated in some spectra. FIG. 9C shows a 2D-NMR TOCSY spectrum of IQN17/D10pep1a complex. Cross-peaks linking these four tryptophan protons are indicated, along with specific assignments. The TOCSY mixing time was 42 ms.

FIG. 10 shows the conformation of the D10pep1 peptide as in the complex with IQN17, as determined by X-ray crystallography.

FIGS. 11A through 11V are the PDB file which lists the atomic coordinates for the crystal structure of IQN17, in which residues 0-28 of the A, B and C chains of the IQN17 trimer are derived from GCN4-pI<sub>Q</sub>I sequence (with three mutations), residues 29-45 of the chains A, B, and C are derived from HIV gp41, ordered water molecules are represented as W, and a bound chloride ion as chain I. The PDB file represents a whole trimer in the crystallographic asymmetric unit.

#### DETAILED DESCRIPTION OF THE INVENTION

The gp41 subunit of the HIV-1 envelope protein mediates fusion of viral and cellular membranes. The crystal structure

of the gp41 ectodomain core is a six-helix bundle composed of three helical hairpins, each consisting of an N-helix paired with an antiparallel C-helix (D. C. Chan, D. Fass, J. M. Berger, P. S. Kim, *Cell*, 89:263 (1997), W. Weissenhorn, A. Dessen, S. C. Harrison, J. J. Skehel, D. C. Wiley, *Nature*, 387:426 (1997); K. Tan, J. Liu, J. Wang, S. Shen, M. Lu, *Proc. Natl. Acad. Sci. USA*, 94:12303 (1997). Three N-helices form an interior, trimeric coiled-coil, and three C-helices wrap around the outside of this N-helix coiled-coil along conserved, hydrophobic grooves. This structure likely corresponds to the core of the fusion-active state of gp41 (discussed in D. C. Chan, D. Fass, J. M. Berger, P. S. Kim, *Cell*, 89:263 (1997), and D. C. Chan and Peter S. Kim, *Cell*, 93:681 (1998)) and shows similarity to the proposed fusogenic structures of envelope fusion proteins from influenza (P. A. Bullough, F. M. Hughson, J. J. Skehel, D. C. Wiley, *Nature*, 371:37 (1994)), Moloney Murine Leukemia Virus (D. Fass, S. C. Harrison, P. S. Kim, *Nat. Struct. Biol.*, 3:465 (1996)), and simian immunodeficiency virus (SIV) (V. N. Malashkevich, D. C. Chan, C. T. Chutkowski, P. S. Kim, *Proc. Natl. Acad. Sci. USA*, 95:9134 (1998), M. Caffrey et al., *EMBO J.*, 17:4572 (1998)), and Ebola virus (W. Weissenhorn et al., *Mol. Cell* 2:605 (1998), V. N. Malashkevich et al., *Proc. Natl. Acad. Sci. USA*, 96:2662 (1999)).

Synthetic C-peptides (peptides corresponding to the C-helix), such as DP178 and C34, are potent inhibitors of HIV-1 membrane fusion and are effective against both laboratory-adapted strains and primary isolates (V. N. Malashkevich, D. C. Chan, C. T. Chutkowski, P. S. Kim, *Proc. Natl. Acad. Sci. USA*, 95:9134 (1998), DP178 corresponds to residues 638–673 of HIV-1 gp41 and is acetylated at the amino terminus and amidated at the carboxy terminus (C. T. Wild, D. C. Shugars, T. K. Greenwell, C. B. McDanal, T. J. Matthews, *Proc. Natl. Acad. Sci. USA*, 91:9770 (1994), S. Jiang, K. Lin, N. Strick, A. R. Neurath, *Nature*, 365:113 (1993)). A Phase I clinical trial with the C-peptide DP178 (also called T-20) indicates that it has antiviral activity in vivo, resulting in reduced viral loads (M. Saag, et al., abstract #771 presented at the Infectious Disease Society of America 35th Annual Meeting, San Francisco, Calif., 16 Sep. 1997; Kilby, J. M. et al. *Nature Med.* 4:1302–1307 (1998)). Based on the structural features of the gp41 core, these peptides are thought to act through a dominant-negative mechanism, in which exogenous C-peptides bind to the central coiled-coil of gp41 and lead to its inactivation (D. C. Chan and P. S. Kim, *Cell*, 93:681 (1998); R. A. Furuta et al., *Nat. Struct. Biol.*, 5:276 (1998); D. C. Chan, D. Fass, J. M. Berger, P. S. Kim, *Cell*, 89:263 (1997), W. Weissenhorn, A. Dessen, S. C. Harrison, J. J. Skehel, D. C. Wiley, *Nature*, 387:426 (1997); K. Tan, J. Liu, J. Wang, S. Shen, M. Lu, *Proc. Natl. Acad. Sci. USA*, 94:12303 (1997), M. Lu, S. C. Blacklow, P. S. Kim, *Nat. Struct. Biol.*, 2:1075 (1995) and C. H. Chen, T. J. Matthews, C. B. McDanal, D. P. Bolognesi, M. L. Greenberg, *J. Virol.*, 69:3771 (1995)). These peptides likely act on a pre-hairpin intermediate of gp41 that forms when the native gp41 structure (i.e., the nonfusogenic conformation present on free virions) is perturbed by gp120/CD4/coreceptor interactions. This pre-hairpin intermediate is proposed to have an exposed N-coiled-coil, thereby allowing C-peptides to bind and inactivate gp41 prior to the formation of the fusion-active hairpin structure (D. C. Chan, P. S. Kim, *Cell*, 93:681 (1998)). This model is further supported by immunoprecipitation experiments indicating that the C-peptide DP178 binds to gp41 (R. A. Furuta, C. T. Wild, Y. Weng, C. D. Weiss, *Nat. Struct. Biol.*, 5:276 (1998)). In addition, viruses escaping DP178 inhibition show mutations in the central coiled-coil region of gp41 (L. T. Rimsky, D. C. Shugars, T. J. Matthews, *J. Virol.*, 72:986 (1998)).

Recent crystallographic studies of gp41 facilitate the development of small-molecule peptidomimetic drugs which, in contrast to C-peptides, have the potential to be orally administered. Within each coiled-coil interface is a deep cavity, formed by a cluster of residues in the N-helix coiled-coil, that is an attractive target for the development of antiviral compounds. Three residues from the C-helix (Trp<sup>628</sup>, Trp<sup>631</sup>, and Ile<sup>635</sup>) insert into this cavity and make extensive hydrophobic contacts. Mutational analysis indicates that two of the N-helix residues (Leu<sup>568</sup> and Trp<sup>571</sup>) comprising this cavity are critical for membrane fusion activity (J. Cao, et al., *J. virol.*, 67:2747 (1993)). Therefore, it is reasonable to expect that compounds that bind with high affinity to this cavity and prevent normal N- and C-helix pairing will be effective HIV-1 inhibitors. In addition, residues in the cavity are highly conserved among diverse HIV-1 isolates. Because of the high structural conservation, drugs targeting this site would have broad activity against diverse HIV-1 isolates, and possibly HIV-2 isolates.

Although this hypothesis is tempting, until now, it had not been demonstrated that these cavity contacts are important for the potency of the C34 inhibitor. In fact, some C-peptides that lack the cavity-binding residues, such as DP178 (C. T. Wild, D. C. Shugars, T. K. Greenwell, C. B. McDanal, T. J. Matthews, *ibid.*, 91:9770 (1994); Kilby, J. M. et al., *Nature Med.*, 4:1302 (1998)), are highly effective inhibitors of HIV-1 membrane fusion. These concerns emphasize the need for systematic structure-function analysis to identify determinants of C-peptide activity.

To determine the role of cavity-contacts in inhibitory activity, structure-based mutagenesis was performed on C34. The core of the gp41 ectodomain (FIG. 1) was reconstituted with two synthetic peptides called N36 and C34 (M. Lu, P. S. Kim, *J. Biomol. Struct. Dyn.*, 15:465 (1997), D. C. Chan, D. Fass, J. M. Berger, P. S. Kim, *Cell*, 89:263 (1997)). Variants of the C34 peptide with single alanine substitutions were synthesized, and the helical content and thermal stability of mutant N36/C34 complexes were quantitated by circular dichroism. As expected, mutation of C34 residues (Met<sup>629</sup>, Arg<sup>633</sup>) that do not contact the N36 coiled-coil had little effect on mean residue ellipticity at 222 nm (a measure of helical content) or stability of N36/C34 complexes (Table 1). However, mutation of the three residues (Trp<sup>628</sup>→Ala, Trp<sup>631</sup>→Ala or Ile<sup>635</sup>→Ala) that project into the N36 coiled-coil cavity resulted in N36/C34 complexes with substantially decreased mean ellipticity and stability (Table 1). The greatest destabilization was observed with the mutant Trp<sup>631</sup>→Ala, which formed N36/C34 complexes with an apparent melting temperature ( $T_m$ ) of 37° C., compared to 66° C. for wildtype. These results demonstrate that C34 residues making hydrophobic contacts with the N36 coiled-coil cavity are important for stabilizing the helical-hairpin structure of the gp41 ectodomain core.

To determine the importance of these residues in the ability of C34 to inhibit membrane fusion, the activity of C34 peptides was tested in HIV-1 viral entry and syncytium assays (Table 1). Mutations (Met<sup>629</sup>→Ala and Arg<sup>633</sup>→Ala) that had little effect on the stability of the N36/C34 complex also had little effect on the inhibitory activity of wildtype C34 (IC<sub>50</sub>~2.1 nM and ~0.55 nM for viral entry and syncytium formation, respectively). However, mutation of the strictly conserved Trp<sup>628</sup> or Trp<sup>631</sup> to alanine resulted in a substantial decrease in activity of ~5 fold and ~30-fold, respectively (Table 1). Mutation of the less well-conserved Ile<sup>635</sup> resulted in only a ~2-fold decrease in inhibitory activity. These results demonstrate for the first time, the C34 residues which make contact with gp41 pocket are important for the inhibitory potency of C34.



The relationship between the potency of mutant C34 peptides and the stability of mutant N36/C34 complexes was clarified by taking advantage of the greatly destabilizing effect of the Trp<sup>631</sup> mutation to construct a series of N36/C34 complexes with a gradation of stabilities. The Trp<sup>631</sup> position was used as a "guest site" and the tryptophan was substituted with natural and artificial amino acids representing a broad range of hydrophobic bulk. In order of increasing hydrophobic bulk, the substitutions were: glycine (Gly), alanine (Ala), L- $\alpha$ -aminobutyric acid (Abu), valine (Val), leucine (Leu), phenylalanine (Phe), the wildtype residue tryptophan (Trp), and L- $\beta$ -(1-naphthyl) alanine (Nal). This approach resulted in a set of C34 peptides that form N36/C34 complexes with  $T_m$ s ranging from 37° C. to 66° C. The  $T_m$ s and  $[\theta]_{222}$  ( $10^3$  deg cm<sup>2</sup> dmol<sup>-1</sup>) for the N36/C34 variants (with IC<sub>50</sub> values (nanomolar) for virus entry and cell fusion, respectively, in parentheses) are: Trp<sup>631</sup>→Gly, 35° C., 17.1 (38±6.1, 25±3.8); Trp<sup>631</sup>→Ala, 37° C., -24.9 (40±4.3, 15±0.8); Trp<sup>631</sup>→Abu, 43° C., -23.2 (16±4.8, 6.9±0.4); Trp<sup>631</sup>→Val, 43° C., -23.9 (13±2.8, 4.5±0.09); Trp<sup>631</sup>→Leu, 50° C., -26.7 (5.3±1.0, 3.2±0.1); Trp<sup>631</sup>→Phe, 59° C., -26.3 (3.6±0.8, 1.6±0.05); wildtype, 66° C., -31.7 (1.5±0.2, 0.55±0.03); Trp<sup>631</sup>→Nal, 62° C., -32.0 (1.4±0.3, 0.79±0.08). The concentration of the Trp<sup>631</sup>→Nal peptide was measured by Nal absorbance using the extinction coefficient  $\epsilon$ =6900 at 282 nm (J. Blake, C. H. Li, *J. Med. Chem.*, 18:423-426 (1975)). In HIV-1 infection and syncytium assays, this series of peptides showed potencies that closely correlated with the  $T_m$  of the corresponding N36/C34 complex (FIG. 2). The potency order of these mutants is wt-Nal>Phe>Leu>Val>Abu>Ala>Gly, in close agreement with the hydrophobic bulk of the substitution and the stability of N36/C34 complexes. There is a striking linear relationship when the IC<sub>50</sub> is plotted on a logarithmic scale as a function of the  $T_m$  (FIG. 2). Since  $\Delta G = -RT \ln K$  ( $\Delta G$ , change in free energy; R, gas constant; T, absolute temperature; and K, equilibrium constant) and  $\Delta T_m$  ( $T_m$ , wildtype complex- $T_m$ , mutant complex) is proportional to  $\Delta(\Delta G)$  ( $\Delta G$ , wildtype complex- $\Delta G$ , mutant complex) (W. J. Becktel, J. A. Schellman, *Biopolymers*, 26:1859 (1987)), the observed linear relationship strongly suggests that the potency of the C34 variants is directly related to their affinity for the N-helix coiled-coil, as predicted by a dominant-negative mode of inhibition. These results provide strong support for the proposal that the coiled-coil cavity in the gp41 core is an attractive drug target. Conserved residues projecting into the hydrophobic cavity clearly play a major role in the ability of C34 to inhibit HIV-1 infection, indicating that this inhibitor works by forming a high-affinity complex with the N-helix coiled-coil. Moving beyond traditional peptides, mirror-image phage display techniques (T. N. Schumacher, et al., *Science*, 271:1854 (1996)), selection-reflection aptamer techniques (K. P. Williams et al., *PNAS*, 94:11285 (1997); S. Klufmann et al., *Nat. Biotech.*, 4:1112 (1996); A. Nolte et al., *Nat. Biotech.*, 14:1116 (1996)), combinatorial chemistry (A. Borchardt, S. D. Liberles, S. R. Biggar, G. R. Crabtree, S. L. Schreiber, *Chem. Biol.*, 4:961 (1997); J. C. Chabala, *Curr. Opin. Biotechnol.*, 6:632 (1995)) and computational approaches in structure-based drug design (H. Kubinyi, *Curr. Opin. Drug Discov. Develop.*, 1:16 (1998)), can be used to identify D-peptides, peptidomimetics, and small molecules that bind with high affinity to the coiled-coil cavity. The close correlation between N36/C34 stability and C34 inhibitory potency suggests that the effectiveness of such compounds will depend critically on the strength of their cavity-contacts. These results suggest that candidate compounds can be tested for the ability to form a stable

complex with N36, thereby providing a basis for rapid, quantitative screens to identify and evaluate potential inhibitors of HIV-1 entry.

Small-molecule inhibitors directed against the cavity of the central coiled-coil target one of the most highly conserved regions of the HIV-1 envelope proteins. The analogous cavity in the SIV gp41 core has an essentially identical structure, with conservation of side chain conformations (V. N. Malashkevich, D. C. Chan, C. T. Chutkowski, P. S. Kim, *Proc. Natl. Acad. Sci. USA*, 95:9134 (1998)). This high degree of structural conservation explains the broad neutralizing activity of C-peptides, which are effective against laboratory-adapted strains as well as primary isolates (C. T. Wild, D. C. Shugars, T. K. Greenwell, C. B. McDanal, T. J. Matthews, *Proc. Natl. Acad. Sci. USA*, 91:9770 (1994), S. Jiang, K. Lin, N. Strick, A. R. Neurath, *Nature*, 365:113 (1993)). Remarkably, SIV C34 peptide is nearly as effective as HIV-1 C34 in inhibiting HIV-1 infection (V. N. Malashkevich, D. C. Chan, C. T. Chutkowski, P. S. Kim, *Proc. Natl. Acad. Sci. USA*, 95:9134 (1998)). In addition, a C-peptide (T649) containing the cavity-binding region is much less susceptible to the evolution of resistant virus (L. T. Rimsky, D. C. Shugars, T. J. Matthews, *J. Virol.*, 72:986 (1998)) than DP178 (also called T-20), which lacks this region. These observations are evidence that high-affinity ligands targeting the coiled-coil surface, particularly its cavity, will have broad activity against diverse HIV isolates (including HIV-2) and will be less likely to be bypassed by drug-escape mutants.

These studies on the mechanism of C-peptide action also support the hypothesis that the trimeric hairpin structure of the gp41 core (Chan, D. C. et al., *Cell*, 89:263 (1997); Weissenhorn, W. et al., *Nature*, 387:426 (1997); Tan, K. et al., *Proc. Natl. Acad. Sci. USA*, 94:12303 (1997)) corresponds to the fusion-active state of gp41. The work described herein shows that the inhibitory potency of C34 depends on its ability to bind to the N-coiled-coil of gp41. Since the hairpin structure of gp41 is extremely stable (with a melting temperature in excess of 90° C.) (Lu, M. et al. *Nat. Struct. Biol.*, 2:1075 (1995)), it is unlikely that nanomolar concentrations of C34 can disrupt this structure once it has formed, especially given the high effective concentration of the N- and C-helices within an intact gp41 molecule. Rather, C-peptides likely act prior to the formation of the gp41 hairpin by binding to a transient pre-hairpin intermediate, in which the central coiled-coil is exposed. Binding of C-peptides to this pre-hairpin intermediate inactivates gp41 and prevents its conversion to the fusion-active hairpin structure (D. C. Chan, P. S. Kim, *Cell*, 93:681 (1998)).

As described herein, the pocket on the surface of the N-helix coiled-coil of HIV-1 envelope protein gp41 subunit is a drug target. Similarly, cavities on other pathogens (e.g., HIV-2) which can cause AIDS or on pathogens which cause AIDS-like conditions in nonhuman mammals (e.g., SIV) are also drug targets. As described herein, available methods (e.g., mirror image phage display methods, combinatorial chemistry, computational approaches and other drug screening and medicinal chemistry methods) can be used to identify peptides, D-peptides, peptidomimetics and small molecules that bind the coiled-coil cavity of HIV-1 (and/or HIV-2) with sufficient affinity to interfere with viral entry into cells and, thus, inhibit viral infection. As further described herein (Example 3), mirror image phage display has been used to identify D-peptides which bind to a cavity on the surface of the N-helix coiled-coil of HIV-1 gp41.

As a result of the work described herein, screening assays which identify molecules or compounds (agents or drugs)



that prevent C34/N36 complex formation and/or disrupt the complex once it has formed are available, as are methods of identifying molecules or compounds (agents or drugs) which bind the N-helix coiled-coil pocket of HIV gp41. Such drugs or agents are useful to inhibit (totally or partially) HIV entry into cells and, thus, infection by HIV.

Methods of screening for compounds or molecules (referred to as drugs or agents) that interfere with formation of a stable complex between C34 and N36 or disrupt a complex between the two and methods of screening for compounds or molecules that bind the N-helix coiled-coil pocket of HIV gp41 are a subject of the present invention.

In one embodiment of a screening method of the present invention, drugs which interfere with formation of a complex between C34 peptide and N36 peptide are identified by combining a candidate drug (a compound or molecule to be assessed for its ability to interfere with formation of a complex between C34 and N36) with C34 and N36, thus forming a test sample, under conditions appropriate for formation of a complex between C34 and N36 and determining whether formation of C34/N36 complex is inhibited (partially or totally) in the test sample. Results of this assessment can be compared with the results of an appropriate control, which is the same combination as the test sample, except that the candidate drug is not present; the control is subjected to the same conditions as is the test sample. If C34/N36 complex is not formed or is formed to a lesser extent in the presence of the candidate drug (in the test sample) than in its absence, the candidate drug is a drug that interferes with formation of a stable complex between C34 and N36. Such a drug is also referred to herein as an inhibitor of C34/N36 complex formation. Inhibition of complex formation can be assessed by determining the extent to which binding of the two members of the complex occurs, such as by means of a fluorescence assay (e.g., FRET), in which C34 and N36 are each labeled by a member of a pair of donor-acceptor molecules or one end of one of the peptides (e.g., the N-terminus of C34) is labeled with one member of such a pair (EDANS) and the natural fluorophore tryptophan, present in the N36 peptide, is the other member of the donor/acceptor pair. Binding of the C34 and N36 is assessed by the extent to which light emission (FRET) occurs from the acceptor model and/or the wavelength spectrum of the light emitted is altered. Prevention of binding by the candidate drug alters the extent to which light is emitted and/or prevents the shift in wavelength that would occur if binding of C34 and N36 occurred. Alternatively, C34 can be labeled with a detectable label, such as a radiolabel (e.g., by synthesizing a variant C34 with a kinase recognition site that can be labeled with a kinase and radioactive ATP). The radiolabeled C34 and the candidate drug are combined with N36 immobilized to, for example, a solid surface (e.g., a bead or a plastic well), thus producing a test sample. The extent to which binding of labeled C34 with immobilized N36 occurs is determined and compared with the extent to which binding of labeled C34 to immobilized N36 occurs under the same conditions to which the test sample is subjected, but in the absence of the candidate drug (in a control sample). Typically, this assessment is carried out after the sample has been maintained for sufficient time and under appropriate conditions for C34/N36 binding to occur and a subsequent wash to remove any unbound C34 and candidate drug. If binding occurs in the test sample to a lesser extent than in the control sample, as evidenced by less radiolabel bound to the immobilized N36 in the test sample than in the control sample, the candidate drug is an inhibitor of binding of C34 and N36.

Alternatively, the label or tag on C34 can be a member of a binding pair, the other member of which is used to detect binding to N36. For example, C34 can be tagged with biotin (through standard solid-state peptide synthesis, for example) and combined with N36, which can be in solution or bound to a solid surface, such as a bead, well or flat/planar surface, along with the candidate drug (test sample) or in the absence or the candidate drug (control sample). Binding of C34 to N36 is assessed by detecting the presence of biotin associated with N36, such as through the use of labeled streptavidin (e.g., streptavidin-HRP, streptavidin-AP or iodinated streptavidin), which binds the biotin on C34 and is then itself detected through its label. If binding occurs less in the presence of the candidate drug (in the test sample) than in the absence of the candidate drug (in the control sample), as indicated by the presence of less biotin detected on N36 in the test sample than in the control sample, the candidate drug is an inhibitor of C34/N36 binding. The candidate drugs can be obtained, for example, from a library of synthetic organic compounds or random peptide sequences, which can be generated synthetically or through recombinant technology.

In a similar fashion, the ability of a candidate drug to disrupt C34/N36 binding can be assessed, to identify inhibitors of C34/N36 and, thus, of HIV infection. In this embodiment, preformed C34/N36 complex is combined with a candidate drug, which is to be assessed for its ability to disrupt the complex, thus producing a test sample. The control sample is the same as the test sample, except that the control sample does not contain the candidate drug; it is treated in the same manner as the test sample. If C34/N36 binding is disrupted in the presence of the candidate drug and not in the control sample or if disruption of the complex occurs to a greater extent in the test sample than in the control sample, the candidate drug is an inhibitor (disrupter) of C34/N36. Detection of disruption of binding can be carried out as described above for detection of/prevention of/interference with binding of C34/N36 (e.g., by FRET or a fluorescence assay, by detecting a radiolabel or other detectable label, such as biotin.)

Results described herein demonstrate that hybrids (i.e., fusion proteins) can be made between a trimeric version of the coiled-coil region of a protein (such as GCN4) and the N-helix coiled-coil of HIV gp41, and that such hybrids are trimeric (i.e., not aggregated) and 100% helical. Results described herein also clearly indicate that such fusion proteins do not disrupt or alter the structure of the N-peptide large cavity (i.e., hydrophobic pocket), which is essentially the same in IQN17 (ligand-free and in complex with D10pep1; see Example 5) as it is in N36 (i.e., in complex with C34; Chan D. C. et al. *Cell*, 89, 263 (1997)).

FIGS. 5A, 5B and 6 present results of assessment of peptides described herein. In FIGS. 5A-5B, the IQN17 crystal structure is shown to be a continuous, three-stranded coiled-coil; the 17 residues derived from HIV gp41 form a hydrophobic pocket very similar to that found in the crystal structure of gp41. As shown, D10pep1 is bound to this pocket and the residues of D10pep1 that correspond to the conserved residues (leucine, tryptophan, tryptophan) found in all of the D-peptide inhibitors described herein are packed into this pocket, clearly indicating that other D-peptide inhibitors which comprise these conserved residues would bind to IQN17 in the same manner. FIG. 6 shows results of syncytia assays carried out according to the method described by Chan et al. (Chan, D. C. et al. *Proc. Natl. Acad. Sci.*, 95: 15613-15617 (1998)). In the experiments whose results are represented in FIG. 6, D-peptides identified as described herein were used. In each instance, a blocking

group (e.g., an acetyl group) was present at the N terminus and a CONH<sub>2</sub> (amide) was present at the C-terminus. Results of these assays showed a range of IC<sub>50</sub> concentrations, where IC<sub>50</sub> is the concentration at which one half of the number of syncytia are observed, compared to the control, in which no peptide is included. For example, D10pep5 with two lysines at the N-terminus has an IC<sub>50</sub> of approximately 6 μM.

In another embodiment, the invention relates to a method of identifying a drug that binds the N-helix coiled-coil cavity of HIV gp41. Here, too, the assay is based on assessing loss or decrease in binding, but unlike the C34/N36 complex assay described above, which is a more general assay in that it covers or detects interaction with any portion of the groove formed by the N-helical region of HIV gp41, this embodiment focuses on the HIV gp41 hydrophobic pocket (the N-helix coiled-coil cavity). In this embodiment, the method comprises combining a candidate drug to be assessed for its ability to bind the N-helix coiled-coil cavity of HIV gp41 with a fusion protein that comprises a trimeric version of the coiled-coil region of a protein and a sufficient portion of the N-peptide of HIV gp41 to include the HIV gp41 cavity, under conditions appropriate for presentation of the HIV gp41 cavity for binding by a peptide or other molecule and determining (e.g., in a high throughput screen) whether the candidate drug binds the fusion protein. If binding occurs, the candidate drug is a "hit" that may be a drug that binds the N-helix coiled-coil cavity of HIV gp41. If binding occurs, the candidate drug has bound the N-helix coiled coil and it can be determined if it binds to the coiled-coil cavity. Such "hits" can then be screened in secondary assays, such as the cell/cell fusion assay and HIV infectivity assay to determine if the candidate drug is a drug. Alternatively, or in addition, such "hits" can be assessed further by use of a counterscreen with other fusion proteins (or peptides), to which pocket-binding molecules will not bind. For example, GCN4-pI<sub>Q</sub>I (with the same three surface mutations as in IQN17) or a version of IQN17 with a point mutation in the hydrophobic pocket, IQN17(G39W), in which glycine 39 is mutated to tryptophan, resulting in a large protrusion into the pocket, can be used in a counterscreen. In this example, a candidate drug that binds to IQN17 but not to GCN4-pI<sub>Q</sub>I (with the same three surface mutations as in IQN17) or IQN17(G39W) is a drug that binds the N-helix coiled-coil cavity of HIV gp41.

In a further embodiment, a competitive assay is carried out. In this embodiment, a peptide or protein that binds the N-helix coiled-coil cavity of HIV gp41 is combined with the candidate drug and the fusion protein and whether the candidate drug binds the HIV gp41 cavity is determined in the presence of the peptide that binds the N-helix coiled cavity of HIV gp41. If the candidate drug binds the fusion protein, it is a drug that binds the HIV gp41 cavity. For example, a fusion protein which comprises a trimeric version of the coiled-coil region of GCN4 and the C-terminus of the N peptide of HIV gp41 that includes the N-helix coiled-coil cavity (IQN17) is combined with a "reference" D-peptide (e.g., any of the D-peptides described herein or variants thereof) that binds the N-helix coiled-coil cavity and a candidate drug to be assessed for its ability to bind the N-helix coiled-coil cavity of HIV gp41, thus producing a test sample, which is maintained under conditions appropriate for binding of the D-peptide to bind to the cavity. A control sample, which includes the same components as the test sample, except for the candidate drug, and is handled in the same manner as the test sample, is also assessed. In both samples, binding of the reference D-peptide is assessed. If

binding of the reference D-peptide occurs to a lesser extent in the presence of the candidate drug (in the test sample) than in its absence (in the control sample), the candidate drug is a drug that binds the N-helix coiled-coil cavity of HIV gp41. Detection of binding is assessed, for example, in a similar manner as described above for the C34/N36 embodiment of the invention. For example, the D-peptide is labeled with a detectable label, such as a radiolabel or a first member of a binding pair (e.g., biotin), and the extent to which the N-helix coiled-coil cavity bears the label (after the samples have been maintained under conditions appropriate for binding of the reference D-peptide to the cavity) is determined. In the case in which radiolabeling is used, the extent to which the fusion protein bears the radiolabel is assessed in the test sample and compared with the extent to which the fusion protein bears the radiolabel in the control sample. If the detectable label is a first member of a binding pair (e.g. biotin), the second member of the pair (a binding partner) is added to the samples in order to detect the extent to which the fusion protein is bound by the reference D-peptide. This can be done directly or indirectly (e.g., by adding a molecule, such as an antibody or other moiety which binds the second member of the binding pair). Less of the label will be present on the fusion protein (N-helix coiled-coil cavity) if the candidate drug has inhibited (totally or partially) binding of the D-peptide to the cavity. If binding occurs to a lesser extent in the test sample (in the presence of the candidate drug) than in the control sample (in the absence of the candidate drug), then the candidate drug is a drug that binds the N-helix coiled-coil cavity of HIV gp41.

IQN17, or a variant thereof, in the D-enantiomer, is useful to identify molecules or compounds which are members of a library or collection and bind the N-helix coiled-coil of gp41. For example, a library or collection of molecules or compounds, such as a phage display library, can be screened with IQN17 in the D-enantiomer to identify members that bind the pocket. This has been carried out successfully, as described herein. The mirror image of IQN17, or a variant thereof, is used as the target molecule. As used herein, the terms "D-enantiomer of a polypeptide" and "D-peptide" refer to the exact mirror image of the molecule in the natural handedness. Thus, for amino acid residues that contain a second chiral center, such as Ile and Thr, the exact mirror image of the naturally-occurring amino acid residue is used to create the D version of the polypeptide. Also as used herein, the terms "D-amino acids" and "L-amino acids" are both meant to include the non-chiral amino acid glycine. D-IQN17 can be immobilized to a solid surface, such as by addition of one member of a binding pair (e.g., biotin) to it and addition of the other member of the pair (e.g., streptavidin) to the solid surface. Binding of the two members results in immobilization of D-IQN17 on the solid surface, such as for phage panning. A linker which is an enzyme recognition site (e.g., an amino acid linker such as Gly-Lys-Gly, in which an L-lysine residue is used) can be placed between the D-IQN17 sequence and the binding pair member (between the biotin and D-IQN17) to provide an enzyme recognition site (here, a trypsin recognition site), so that bound phage can be eluted by a trypsin digestion, rather than by non-specific elution, such as acid addition. The phage display library can be a library of L-amino acid peptides of any appropriate length fused to an appropriate phage gene. In one embodiment, it is a phage display library of L-amino acid peptides fused to the gIII gene of M13 phage. The peptides, in one embodiment, comprise 10 randomly encoded amino acid residues flanked by either a cysteine or a serine on both sides. Typically, several rounds

of panning are carried out. D-IQN17-specific binding phage are identified. Phage that bind only the gp41 region of D-IQN17 can be identified by post-panning assessment, such as by screening against wells that lack the antigen and then further testing against a panel of molecules. For example, specific pocket-binding phage include those that bind D-IQN17 but not D-GCN4-pL<sub>Q</sub>I (with the same three surface mutations as in IQN17) or a version of D-IQN17 with a point mutation in the hydrophobic pocket, D-IQN17 (G39W), in which glycine 39 is mutated to tryptophan, resulting in a large protrusion into the pocket. D-peptides identified in this manner can be assessed for their ability to inhibit HIV gp41, using known assays, such as the cell/cell fusion assay and HIV infectivity assay. The mirror-image phage display method described herein has demonstrated the value of IQN17 and IQN17(G39W), and their D-enantiomers in identifying inhibitors of HIV-1 entry that bind the gp41 pocket. Of nine specific pocket-binding phage sequences identified (phage that bind to D-IQN17 but not to D-IQN17(G39W)), eight contain a consensus EWXWL sequence and inhibit HIV-1 gp41-induced syncytia formation when tested as D-peptides. The ninth peptide was toxic to cells and was not investigated further.

The D-versions of IQN17 and IQN17(G39W) can be used in a similar manner with other biologically encoded libraries, to discover other pocket-binding molecules that are not subject to enzymatic degradation by natural enzymes. For example, other phage-display libraries can be used to identify new D-peptide inhibitors (e.g., with a different number of residues between the flanking Cys residues and/or with randomly encoded amino acid residues outside the regions flanked by cysteine residues and/or with more than two cysteine residues). Strategies for encoding peptide libraries without phage (e.g., in which the encoding mRNA is attached to the peptide) can be used to identify D-peptide inhibitors. RNA or DNA libraries can be used (e.g., with SELEX methods) to identify L-ribose- or L-deoxyribose-based RNA or DNA aptamers, respectively, that bind to the hydrophobic pocket and are not substrates for natural nucleases (see e.g., Williams et al., *PNAS*, 74:11285 (1997)).

Although the versions of IQN17 and IQN17(G39W) of natural L-handedness can also be used in similar manner with biologically encoded libraries, the most likely applications will be with other, non-biologically encoded libraries. For example, chemical combinatorial libraries on beads (of the one-bead, one-compound variety) can be screened with labeled IQN17 (e.g., radioactive or with a chromophore) to identify beads containing molecules that bind to IQN17. In this example, IQN17(G39W) can be used as a counterscreen to determine if the molecules on the bead bind to the pocket of IQN17. (If they bind to IQN17(G39W), then they are not likely to be pocket-binding molecules.) As another example, beads to which IQN17 had been previously attached can be incubated with a mixture of potential pocket-binding molecules (e.g., a mixture of chemicals, or a natural product extract). IQN17 (bound to the beads) can then be separated from the mixture, washed, and then subjected to conditions (e.g., organic solvent, low pH, high temperature) that elute molecules bound to the IQN17 on the beads. The eluted molecules (i.e., potential pocket-binding molecules) could be identified by analytical chemistry methods (e.g., HPLC, mass spectrometry). A counterscreen with IQN17(G39W) is useful to help to identify true pocket-binding molecules.

Drugs identified by the methods described above are then further tested for their ability to inhibit (totally or partially) HIV gp41 function (membrane fusion) and, thus entry into cells, using further in vitro assays, such as the syncytium

assays and/or infectivity assays described herein or others known to those of skill in the art, and/or in vivo assays in appropriate animal models or in humans.

One embodiment of the present invention is a method of identifying a drug that binds the N-helix coiled-coil of HIV gp41, particularly the N-helix coiled-coil pocket. The method comprises combining a candidate drug to be assessed for its ability to bind the N-helix coiled-coil pocket of HIV gp41 and peptide which comprises a soluble, trimeric coiled-coil and a sufficient portion of the N-peptide of HIV gp41 to include the HIV gp41 pocket, under conditions appropriate for presentation of the HIV gp41 pocket for binding by a molecule or compound (e.g., a drug) and determining whether the candidate drug binds the HIV gp41 pocket. If binding of the candidate drug with the HIV gp41 pocket occurs, the candidate drug is a drug which binds the N-helix coiled-coil pocket of HIV gp41. Optionally, binding of the candidate drug can be assessed in the assay as described above, except that a peptide that binds the N-helix coiled-coil pocket (a peptide previously identified as one which binds the pocket) is combined with the candidate drug and the peptide. In this competitive assay, binding of the candidate drug to the N-helix coiled-coil pocket is assessed in the presence of a known binding moiety (a molecule or compound which binds the pocket). If binding of the candidate drug occurs in the presence of the known binding moiety, the candidate drug is a drug which binds the N-helix coiled-coil pocket with sufficient affinity to successfully compete with the known binding moiety. The fusion protein used in this embodiment comprises a soluble, trimeric version of a coiled-coil, such as a soluble, trimeric version of the coiled-coil region of a protein (e.g., a non-HIV protein, such as that of GCN4 or GCN4-pL<sub>Q</sub>I, although an HIV protein can be used) and a sufficient portion of the N-peptide of HIV gp41 to include the HIV gp41 cavity. For example, this portion can comprise SEQ ID NO.: 20 or a sufficient portion to comprise the cavity and, when present in an appropriate fusion protein or other soluble model, present the cavity in such a manner that it is available for binding. Alternatively, a variant of the HIV gp41 sequence present herein, a sequence from another strain of the human virus (e.g., HIV-2) or a sequence from another species (e.g., SIV, feline immunodeficiency virus, Visna virus (M. Singh et al., *J. Mol. Biol.*, 290:1031 (1999)) can be used in the fusion protein or soluble model. The fusion protein can comprise a soluble, trimeric version of the coiled-coil of any protein, provided that when it is in the fusion protein with the HIV component, the HIV cavity is presented in such a manner that it is available for binding. It can be, for example, that of GCN4-pL<sub>Q</sub>I, GCN4-pII, Moloney Murine Leukemia Virus (Mo-MLV) or the ABC heterotrimer. In one embodiment, the fusion protein is IQN17 in the D-form. In another embodiment, the fusion protein is IQN17 in the natural L-handedness.

In the competitive assay format, any peptide known to bind the N-helix coiled-coil cavity can be used as the known binding moiety. For example, any of the peptides described herein (SEQ ID NOS.: 3-12, 15, 17-19, 23, 24) or a variant or portion thereof can be used. Also, any non-peptide pocket-binding molecule can be used in the competitive assay format. The competitive assay can be performed in solution, on a bead, or on a solid surface.

In one embodiment, the candidate drug is detectably labeled and binding of the candidate drug to the HIV gp41 N-helix coiled-coil is determined by detecting the presence of the detectable label on the HIV gp41 N-helix coiled-coil (as a result of binding of the labeled candidate drug to the

N-helix coiled-coil). Detection of the label on the helix coiled-coil pocket of the soluble model is indicative of binding of the candidate drug to the N-helix coiled-coil pocket and demonstrates that the candidate drug is a drug which binds the N-helix coiled-coil pocket. If the labeled candidate drug is detected on the fusion protein, the candidate drug is a drug which binds the N-helix coiled-coil cavity.

In another embodiment of the method of identifying a drug that binds the N-helix coiled-coil pocket of the HIV gp41, a soluble model that presents the pocket in such a manner that it is available for binding by a drug is combined with a candidate drug and whether binding of the candidate drug with the N-helix coiled-coil of the soluble model occurs is determined. If binding occurs, the candidate drug is a drug which binds the pocket. Here, too, a competitive assay format can be used. The components of the competition assay (e.g., IQN17 and a D-peptide) can be labeled, with any of a variety of detectable labels, including fluorophore/quencher combinations. The candidate drug can be labeled, as described above, with any of a variety of detectable labels. The components of the soluble model (fusion protein) used in this embodiment and the competing moiety which is used in a competitive assay format can also be as described above.

The present invention also relates to a method of producing a drug that binds the N-helix coiled-coil pocket of HIV gp41. In one embodiment, the method is carried out as follows: A soluble model that presents the N-helix coiled-coil pocket of HIV gp41 or a fusion protein which comprises a soluble, trimeric coiled-coil (e.g., of a protein, such as a non-HIV protein, such as GCN4-pI<sub>Q</sub>I, GCN4-pII, Mo-MLV, ABC heterotrimer or an HIV protein) is combined with a candidate drug to be assessed for its ability to bind the N-helix coiled-coil pocket of HIV gp41 and inhibit entry into cells, under conditions appropriate for presentation of the HIV gp41 pocket for binding by a drug. Whether the candidate drug binds the HIV gp41 pocket is determined, wherein if binding of the candidate drug to the N-helix coiled-coil pocket of HIV gp41 occurs, the candidate drug is a drug which binds the N-helix coiled-coil cavity of HIV gp41. In this embodiment, the fusion protein comprises a soluble, trimeric coiled-coil (e.g., of a protein such as a non-HIV protein, such as a soluble, trimeric coiled-coil of GCN4, GCN4-pI<sub>Q</sub>I, GCN4-pII, Mo-MLV, ABC heterotrimer or an HIV protein) and a sufficient portion of the N-peptide of HIV gp41 to include the HIV gp41 N-helix coiled-coil pocket (e.g., all or a portion of SEQ ID NO.: 20, a variant or modification thereof or a sequence from another strain or species). IQN17, described herein, can be used in this method; the D enantiomer of IQN17 can also be used (e.g., in mirror-image phage applications). The ability of the drug produced to inhibit HIV entry into cells is assessed, for example, in a syncytium assay and/or an infectivity assay, as described herein. It can be further assessed in an appropriate animal model or in humans.

The invention also relates to a method of producing a drug that binds the N-helix coiled-coil pocket of HIV gp41. The method comprises: producing or obtaining a soluble model of the N-helix coiled-coil pocket of HIV gp41 (e.g., a fusion protein as described herein and particularly IQN17 or a variant thereof); combining a candidate drug (a molecule or compound) to be assessed for its ability to bind the N-helix coiled-coil pocket of HIV gp41 and the soluble model of the N-helix coiled-coil pocket of HIV gp41 and determining whether the candidate drug binds the N-helix coiled-coil pocket of HIV gp41. If the candidate drug binds the N-helix

coiled-coil pocket of HIV gp41, the candidate drug is a drug which binds the N-helix coiled-coil pocket of HIV gp41; as a result, a drug which binds the N-helix coiled-coil cavity of HIV gp41 is produced. The fusion protein used in this embodiment is described herein and can be, for example, IQN17, the D enantiomer of IQN17, or variants thereof. Alternatively, a drug that binds the N-helix coiled-coil pocket of HIV gp41 and inhibits entry of HIV into cells can be produced by a method comprising: producing or obtaining a soluble model of the N-helix coiled-coil pocket of HIV gp41, as described herein; combining the soluble model and a candidate drug to be assessed for its ability to bind the N-helix coiled-coil pocket of HIV gp41; determining whether the candidate drug binds the N-helix coiled-coil pocket of the soluble model (fusion protein), wherein if binding occurs, the candidate drug is a drug which binds the N-helix coiled-coil of HIV gp41; and assessing the ability of the drug which binds the N-helix coiled-coil to inhibit HIV entry into cells, wherein if the drug inhibits HIV entry into cells, it is a drug which binds the N-helix coiled-coil pocket of HIV gp41 and inhibits HIV entry into cells. Its ability to inhibit HIV entry into cells can be assessed in vitro (e.g., in a syncytium assay, an infectivity assay) or in vivo (e.g., in an appropriate animal model or in humans). The soluble model can be a peptide which comprises a soluble, trimeric coiled-coil, such as that of a protein (e.g., GCN4-pI<sub>Q</sub>I) and a sufficient portion of the N-peptide of HIV gp41 to include the HIV gp41 pocket.

Drugs identified or produced by the methods described herein, as well as by other methods, which bind the N-helix coiled-coil pocket of HIV gp41 and inhibit HIV entry into cells are also the subject of this invention.

Drugs identified or produced by the methods described herein, as well as by other methods, which bind to more than one N-helix coiled-coil pocket of HIV gp41 and inhibit HIV entry into cells are also the subject of this invention. Such drugs can be obtained, for example, by linking two or more pocket-binding molecules (drugs) via an appropriate linker (e.g., a linker of amino acid residues or other chemical moieties) to increase the effectiveness of inhibition. The pocket-binding molecules that are linked can be the same or different. Drugs identified or produced by the methods described herein or by other methods which bind to the N-helix coiled-coil pocket of HIV gp41, in addition to binding to HIV gp120, CD4, CCR5, CXCR4, or a non-pocket region of HIV gp41 are also the subject of this invention.

Drugs which inhibit HIV gp41 can also be designed or improved with reference to the X-ray crystal structure of the complex between IQN17 and a D-peptide which binds the N-helix coiled-coil cavity presented by IQN17, such as with reference to the X-ray structure of the complex between IQN17 and D10pep1, presented herein. Alternatively, or in addition, drugs which inhibit HIV gp41 can also be designed or improved with reference to the X-ray crystal structure of free IQN17, presented herein.

Compounds and molecules (drugs) identified as described herein inhibit (partially or totally) entry of HIV into cells, and thus are useful therapeutically in uninfected individuals (humans) and infected individuals (e.g., to prevent or reduce infection in an uninfected individual, to reduce or prevent further infection in an infected individual) and as research reagents both to study the mechanism of gp41-induced membrane fusion and to assess the rate of viral clearance by an individual and as reagents to discover or develop other compounds and molecules (drugs) that inhibit entry of HIV into cells. D-peptides described herein (e.g., D10pep5,

D10pep1) have been shown, using the infectivity assay described herein, to inhibit infection of cells. Other D-peptides can be similarly assessed for their ability to inhibit infectivity.

The drugs can be administered by a variety of route(s), such as orally, nasally, intraperitoneally, intramuscularly, vaginally or rectally. In each embodiment, the drug is provided in an appropriate carrier or pharmaceutical composition. For example, a cavity-binding drug can be administered in an appropriate buffer, saline, water, gel, foam, cream or other appropriate carrier. A pharmaceutical composition comprising the drug and, generally, an appropriate carrier and optional components, such as stabilizers, absorption or uptake enhancers, flavorings and/or emulsifying agents, can be formulated and administered in therapeutically effective dose(s) to an individual (uninfected or infected with HIV). In one embodiment, drugs which bind the N-helix coiled-coil of gp41 (e.g., those described herein, DP178 (C. T. Wild, D. C. Shugars, T. K. Greenwell, C. B. McDaniel, T. J. Matthews, *ibid*, 91:9770 (1994)), T649 which corresponds to residues 117-152 of HIV-1 gp41 (HXB2 strain) and is acetylated at the amino terminus and amidated at the carboxy terminus) (L. T. Rimsky, D. C. Shugars, T. J. Matthews, *J. Virol.*, 72:986 (1998), are administered (or applied) as microbicidal agents and interfere with viral entry into cells. For example, a drug or drugs which bind(s) the HIV cavity can be included in a composition which is applied to or contacted with a mucosal surface, such as the vaginal, rectal or oral mucosa. The composition comprises, in addition to the drug, a carrier or base (e.g., a cream, foam, gel, other substance sufficiently viscous to retain the drug, water, buffer) appropriate for application to a mucosal surface or to the surface of a contraceptive device (e.g., condom, cervical cap, diaphragm). The drug can be applied to a mucosal surface, such as by application of a foam, gel, cream, water or other carrier containing the drug. Alternatively, it can be applied by means of a vaginal or rectal suppository which is a carrier or base which contains the drug or drugs and is made of a material which releases or delivers the drug (e.g., by degradation, dissolution, other means of release) under the conditions of use (e.g., vaginal or rectal temperature, pH, moisture conditions). Such compositions can also be administered orally (e.g., swallowed in capsule, pill, liquid or other form) and pass into an individual's blood stream. In other embodiments, controlled or time release (gradual release, release at a particular time after administration or insertion) of the drug can be effected by, for example, incorporating the drug into a composition which releases the drug gradually or after a defined period of time. Alternatively, the drug can be incorporated into a composition which releases the drug immediately or soon after its administration or application (e.g., into the vagina, mouth or rectum). Combined release (e.g., release of some of the drug immediately or soon after insertion, and over time or at a particular time after insertion) can also be effective (e.g., by producing a composition which is comprised of two or more materials: one from which release or delivery occurs immediately or soon after insertion and/or one from which release or delivery is gradual and/or one from which release occurs after a specified period). For example, a drug or drugs which bind the HIV cavity can be incorporated into a sustained release composition such as that taught in U.S. Pat. No. 4,707,362. The cream, foam, gel or suppository can be one also used for birth control purposes (e.g., containing a spermicide or other contraceptive agent), although that is not necessary (e.g., it can be used solely to deliver the anti-HIV drug, alone or in combination

with another non-contraceptive agent, such as an antibacterial or antifungal drug or a lubricating agent). An anti-HIV drug of the present invention can also be administered to an individual through the use of a contraceptive device (e.g., condom, cervical cap, diaphragm) which is coated with or has incorporated therein in a manner which permits release under conditions of use a drug or drugs which bind the HIV gp41 N-helix coiled coil. Release of the drug(s) can occur immediately, gradually or at a specified time, as described above. As a result, they make contact with and bind HIV and reduce or prevent viral entry into cells.

In another embodiment, a drug which interferes with HIV entry into cells by a mechanism other than binding to the gp41 N-helix coiled-coil cavity (e.g., a drug which interferes with viral entry by interfering with gp120 binding at the CD4 stage) is administered or applied to a mucosal surface as described above for drugs which bind to the gp41 N-helix coiled coil.

Fusion proteins of the present invention comprise a soluble, trimeric form or version of a coiled-coil, such as a soluble, trimeric form or version of a coiled-coil region of a protein (of non-HIV origin or of HIV origin) and a sufficient portion of the C-terminal end of the N peptide of HIV gp41 to include (comprise) the HIV coiled-coil cavity or hydrophobic pocket (the pocket-comprising residues of the N-peptide). The N peptide of HIV gp41 can be that of HIV-1, HIV-2, another HIV strain or a strain from another species (e.g., simian immunodeficiency virus (SIV), feline immunodeficiency virus or Visna virus). For example, HIV-2 sequence LLRLTVWGTGNLQARVT (SEQ ID NO: 26), SIV sequence LLRLTVWGTGNLQTRVT (SEQ ID NO: 27) or a sequence comprising invariant residues in HIV-1, HIV-2 and SIV (represented LLXLTVWGXXLQXRX (SEQ ID NO: 42), wherein amino acid residues L, T, V, W, G, K, Q, and R are the single letter code used for amino acid residues and X can be any amino acid residue). Also the subject of this invention is a soluble trimeric model of the HIV gp41 hydrophobic pocket, which can be a D-peptide or an L-peptide and comprises a soluble trimeric coiled coil and a sufficient portion of the N peptide region of HIV gp41 to comprise the amino acid residues which form the pocket of the N-helix coiled-coil region of HIV gp41. The D- or L-peptide can comprise as the soluble, trimeric coiled coil the coiled coil of GCN4-pI<sub>Q</sub>I, of GCN4-pII, of Moloney Murine Leukemia Virus or of the ABC heterotrimer. The component which is a sufficient portion of the N peptide of HIV gp41 to comprise the amino acid residues of the pocket can comprise, for example: LLQLTVWGIKQLQARIL of HIV-1 (SEQ ID NO: 20); LLRLTVWGTGNLQARVT of HIV-2 (SEQ ID NO: 26); LLRLTVWGTGNLQTRVT of SIV (SEQ ID NO: 27) or the invariant residues of these, which are: LLXLTVWGXXLQXRX (SEQ ID NO: 42).

One embodiment of the instant invention are fusion proteins between a trimeric version of the coiled-coil region of a protein (such as GCN4-pI<sub>Q</sub>I) and the N-helix coiled-coil of HIV gp41 that include all, part or none of the N-helix cavity. That is, a fusion protein of the present invention can comprise a trimeric form of the coiled-coil region of GCN4-pI<sub>Q</sub>I and a portion of the N-peptide of HIV-1 gp41, wherein the portion of the N-peptide of gp41 comprises part, or all, or none of the N-helix cavity of HIV-1 gp41. For example, a fusion protein can be made that contains residues from GCN4-pI<sub>Q</sub>I and residues from N36. The fusion protein, denoted IQN24n, contains 29 residues of GCN4-pI<sub>Q</sub>I, including three mutations for increased solubility, and 24 residues from the N-terminal end of N36 (SGIVQQNNLLRAIEAQQHLLQLT) (SEQ ID NO 21);

for recombinant expression in *E. coli*, an extra Met residue is included at the N-terminus. For example, a fusion protein can comprise a portion of the N-peptide of HIV gp41 comprising the amino acid sequence of (SEQ ID.: 21). The sequence of IQN24n is: MRMKQIEDKIEEIESKQKKIE-NEIARIKKLISGIVQQNNLLRAIEAQHLLQLT (SEQ ID.: 22). This fusion protein can be made by a variety of methods, including chemical synthesis or recombinant DNA methods or by recombinant expression in *E. coli*, in which case the N- and C-termini are not blocked. Because the superhelix parameters of the GCN4-pI<sub>Q</sub>I coiled coil are nearly identical to the HIV gp41 N-helix coiled coil, the resulting fusion protein molecule (IQN24n) is predicted to form a long trimeric coiled coil, which presents part of the gp41 N-helix coiled coil as a trimer (not aggregated).

An alternative embodiment of the instant invention provides a method of eliciting an immune response in an individual. The strategy used to create a soluble, trimeric model for part of the gp41 N-terminal region coiled coil is also helpful to develop HIV vaccine candidates. One goal for a potential HIV vaccine is to elicit a neutralizing antibody response that binds to the "pre-hairpin" intermediate of the HIV-1 gp120/gp41 envelope protein complex. In this transient form, the N-helix region of gp41 is exposed, but the C-helix region is not. Although it seems reasonable to use an N-peptide (such as N36, N51 or DP-107) as an immunogen to elicit an antibody response against the N-helix region of gp41, the isolated N-peptides are aggregated and do not properly present the gp41 N-helix coiled-coil trimer. Accordingly, the same strategy described herein to solve this problem for the gp41 hydrophobic pocket can be applied towards the development of soluble, trimeric models of the gp41 N-helix coiled-coil region, in general. Such trimeric models (including IQN17, but also including, for example, peptides that do not contain the pocket residues of gp41) can be used as immunogens to elicit an antibody response to the pre-hairpin intermediate, thereby inhibiting HIV-1 infection. For example, an individual to be immunized can be administered a fusion protein comprising a trimeric form of a coiled-coil region of a protein and a portion of an N-peptide from HIV-1 gp41, wherein the portion from gp41 comprises part of, all of, or none of the N-helix coiled-coil cavity in a pharmaceutically acceptable carrier. For example, IQN24n can be used, either alone or in combination with other materials, in a vaccine, which will elicit the production of antibodies that bind to the coiled coil in the individual to whom it is administered (the vaccinee), and thereby offer protection against infection and/or disease. IQN24n can also be used to identify (from humans, other animals or antibody libraries) and/or raise antibodies (monoclonal and/or polyclonal) that bind to the N-helix coiled coil. This provides the basis for a diagnostic method in which IQN24n (or IQN17 or other soluble trimeric model) is used to assess the presence/absence/level of antibodies that bind the N-helix coiled coil in a biological sample (e.g., blood).

Any of a wide variety of variations can be made in the GCN4-pI<sub>Q</sub>I component of fusion proteins described herein (e.g., IQN17 or IQN24n) and used in the method, provided that these changes do not alter the trimeric state of the coiled-coil. Changes can also be made in the amino acid composition of the fusion protein component which is the portion from the HIV gp41 N36 peptide, to produce variants (e.g., variants of IQN17 or IQN24n). There is no limit to the number or types of amino acid residue changes possible, provided that the trimeric state of the coiled-coil and the structure of the surface of the fusion protein corresponding to the N-peptide coiled coil of HIV gp41 are maintained. The

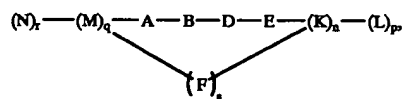
fusion protein component which is the portion of the HIV gp41 N-peptide can include all, part, or none of the N-helix cavity. For example, other parts of N51, N36, DP-107, or other regions of the HIV gp41 N-helix region can be fused to GCN4-pI<sub>Q</sub>I (or another trimeric version of the coiled-coil region of a protein) to generate trimeric (not aggregated) helical coiled-coil fusion proteins and used in the method. There is no limit to the number or types of fusion proteins that can be designed and generated, provided that the trimeric state of the coiled-coil and the structure of the surface of the fusion protein corresponding to the N-peptide coiled coil of HIV gp41 are maintained. Such fusion proteins can be designed and generated using methods known to those of skill in the art, such as evaluating heptad-repeat positions or superhelix parameters of coiled coils.

Described herein are peptides, which can be D-peptides or L-peptides, which bind to a cavity on the surface of the N-helix coiled-coil of HIV envelope glycoprotein gp41 (e.g., HIV-1, HIV-2). Such peptides can be of any length, provided that they are of sufficient length to bind the cavity in such a manner that they interfere with the interaction of the N-helix coiled-coil cavity and amino acid residues of the C-peptide region of HIV gp41 and prevent HIV entry into the cells. For example, D- or L-peptides comprise at least two amino acid residues and generally will be from about two to about 21 amino acid residues. That is, they can comprise any number of amino acid residues from about two to about 21. The amino acid residues can be naturally occurring or non-naturally occurring or modified, as described below. The peptides can be linear or circular.

Examples of D-peptides, identified as described herein, are shown in FIG. 3. Because of library design, each peptide, in addition to the amino acid residues shown, is flanked by GA on the N-terminus and AA on the C-terminus. N-terminal lysine residues were added to improve water solubility.

In one embodiment, the present invention provides compounds which inhibit the binding of the N-helix coiled coil to the C-helix of HIV-1 gp41 envelope protein. Such compounds are of use in a method of treating a patient infected by, or potentially subject to infection by, HIV. These compounds are also of use in a method of assessing the ability of a second compound to bind to the N-helix coiled coil cavity.

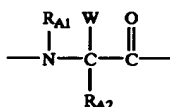
In one embodiment, the compounds which inhibit the binding of the N-helix coiled coil to the C-helix of HIV-1 gp41 envelope protein are of Formula I,



wherein A, B, D and E are each, independently, a D-amino acid residue, an L-amino acid residue, or an N-substituted glycyl residue. Natural or nonnatural amino acid residues can be used. K, L, M and N are each, independently, an amino acid residue or a polypeptide group of from 2 to about 6 amino acid residues which can be the same or different, and n, p, q and r are each, independently, 0 or 1. F is a direct bond or a difunctional linking group and s is 0 or 1.

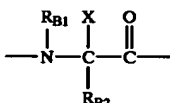
In one subset of the compounds of Formula I, A is a D-amino acid residue, an L-amino acid residue or an N-substituted glycyl residue of the formula

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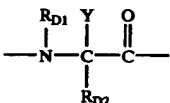
where one of  $R_{A1}$  and  $R_{A2}$  is a substituted or unsubstituted aryl, heteroaryl, arylmethyl, heteroarylmethyl, benzo-fused aryl, benzo-fused heteroaryl, benzo-fused arylmethyl, benzo-fused heteroarylmethyl, cycloalkyl or bicycloalkyl; and the other is hydrogen. W is hydrogen, methyl, trifluoromethyl or halogen, for example, fluorine, chlorine, bromine or iodine.

B is a glycyl residue or D-amino acid or N-substituted glycyl residue of the formula



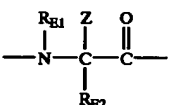
where one of  $R_{B1}$  and  $R_{B2}$  is a substituted or unsubstituted linear, branched or cyclic alkyl, aryl, arylalkyl, heteroaryl or heteroarylalkyl group; and the other is hydrogen X is hydrogen, methyl, trifluoromethyl or halogen, such as fluorine, chlorine, bromine or iodine.

D is a D-amino acid residue or N-substituted glycyl residue of the formula



where one of  $R_{D1}$  and  $R_{D2}$  is a substituted or unsubstituted aryl, heteroaryl, arylmethyl, heteroarylmethyl, benzo-fused aryl, benzo-fused heteroaryl, benzo-fused arylmethyl; benzo-fused heteroarylmethyl, cycloalkyl or bicycloalkyl; and the other is hydrogen. Y is hydrogen, methyl, trifluoromethyl or halogen, such as fluorine, chlorine, bromine or iodine.

E is a D-amino acid residue or N-substituted glycyl residue of the formula



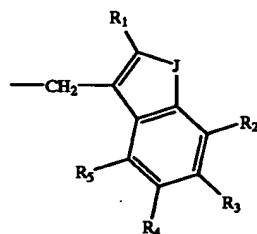
where one of  $R_{E1}$  and  $R_{E2}$  is a substituted or unsubstituted, linear, branched or cyclic alkyl, aryl or arylalkyl group; and the other is hydrogen. Z is hydrogen, methyl, trifluoromethyl or halogen, such as fluorine, chlorine, bromine or iodine.

K, L, M and N are each, independently, composed of from 1 to about 6 (which can be the same or different), D-amino acid residues, L-amino acid residues, N-substituted glycyl residues or a combination thereof. Natural or nonnatural amino acid residues can be used. One or more of the amino acid residues or N-substituted glycyl residues can, optionally, be substituted at the  $\alpha$ -carbon by a methyl or trifluoromethyl group, or a halogen, such as a fluorine, chlorine, bromine or iodine atom.

In a preferred embodiment, one of  $R_{A1}$  and  $R_{A2}$  and one of  $R_{D1}$  and  $R_{D2}$  are, independently, a phenyl, substituted phenyl, naphthyl, substituted naphthyl, naphthylmethyl,

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substituted naphthylmethyl, benzyl or substituted benzyl group, or a group of the formula



where J is O, S or NR, where R is H or linear, branched or cyclic  $C_1$ - $C_6$ -alkyl, preferably methyl.  $R_1$ ,  $R_2$ ,  $R_3$ ,  $R_4$  and  $R_5$  are independently selected from the group consisting of hydrogen, halogen and alkyl, preferably, linear, branched or cyclic  $C_1$ - $C_4$ -alkyl, such as methyl. Suitable phenyl, naphthyl, naphthylmethyl and benzyl substituents include alkyl, preferably linear, branched or cyclic  $C_1$ - $C_4$ -alkyl, such as methyl; and halogen, such as fluorine, chlorine, bromine or iodine. More preferably,  $R_{A1}$  and  $R_{D1}$  are both hydrogen, and  $R_{A2}$  and  $R_{D2}$  are each, independently, one of the foregoing groups.

Preferably, one of  $R_{B1}$  and  $R_{B2}$  is hydrogen, substituted or unsubstituted linear, branched or cyclic  $C_1$ - $C_4$ -alkyl, phenyl, benzyl, naphthyl or naphthylmethyl. Suitable substituents include linear, branched or cyclic  $C_1$ - $C_4$ -alkyl groups and halogens, such as fluorine, chlorine, bromine or iodine. More preferably,  $R_{B1}$  is hydrogen and  $R_{B2}$  is one of the foregoing groups.

Preferably, one of  $R_{E1}$  and  $R_{E2}$  is a substituted or unsubstituted, linear, branched or cyclic  $C_1$ - $C_6$ -alkyl group or a substituted or unsubstituted phenyl or naphthyl group. Suitable substituents include linear, branched or cyclic  $C_1$ - $C_4$ -alkyl groups, such as methyl, and halogens, such as fluorine, chlorine, bromine and iodine. More preferably,  $R_{E1}$  is hydrogen and  $R_{E2}$  is one of the foregoing groups.

In a preferred subset of the compounds of formula I, A and D are each a D-tryptophan residue and E is a D-leucine residue.

Preferably, K is a D-amino acid residue or an N-substituted glycyl residue comprising an amino-, carboxyl- or sulfhydryl substituted side chain, such as a cysteine, glutamic acid, aspartic acid or lysine residue, and

L is a polypeptide comprising 2 or 3 D-amino acid residues, L-amino acid residues (the D- or L-amino acid residues can be the same or different) or N-substituted glycine residues. For example, in one embodiment, L comprises 2 or 3 residues selected from among D-glycine, D-alanine or D- $\alpha$ - $C_1$ - $C_4$ -alkylglycine.

Preferably, M is a polypeptide group comprising from 2 to about 8 D-amino acid residues, of which at least one comprises an amino-, carboxy- or sulfhydryl substituted side chain, such as a cysteine, glutamic acid, aspartic acid or lysine residue. N is, preferably, a polypeptide group comprising from 1 to about 6 amino acid residues, of which at least one is a lysine residue.

The identity of divalent linking group F is not critical, as long as it is of a suitable length to position residues A to E to interact with the N-helix coiled coil cavity (J. R. Morphy, *Curr. Op. Drug Discov. Develop.*, 1:59-65 (1998)). For example, F preferably has a length from about 2 to about 40 atoms. In one embodiment, F is a direct bond or a polypeptide linking group of the formula  $-P_n-$ , wherein n is 1 to about 12 and each P is independently an L- or D-amino acid or N-substituted glycyl residue, a glycyl residue or an N-substituted glycyl derivative.



In another embodiment, F is a substituted or unsubstituted  $C_4-C_{40}$ -alkylene group, such as a polymethylene group of the formula  $-(CH_2)_m-$ , wherein m is from about 4 to about 40; an alkylene group which is interrupted at one or more points by a heteroatom, such as a nitrogen, oxygen or sulfur atom. For example, F can be a group  $(CH_2CH_2O)_q-$ , wherein q is from 1 to about 20. F can also be an alkylene group which is interrupted at one or more points by a phenylene or heteroarylene group, or a polysaccharide group, for example, a glycoside or poly(glycoside) group comprising one or more glycoside groups, for example, from 1 to about 10 glycoside groups. Suitable glycosides include glucoside, lactoside, mannoside, galactoside, fucoside, fructoside, guloside, alloside, altroside, taloside, idoside and others, such as pyranosides and furanosides, which are known in the art.

In compounds of Formula I having a C-terminal amino acid residue, the C-terminal residue can be, for example, in the form of an amide, an N-substituted amide or a carboxylic acid protecting group, as is known in the art. The nitrogen atom of an N-terminal residue can be acylated, for example, acetylated, or substituted with an amino protecting group, as is known in the art.

The term "D-amino acid residue", as used herein, refers to an  $\alpha$ -amino acid residue having the same absolute configuration as D-glyceraldehyde. When the amino acid residue includes a first non-hydrogen  $\alpha$  substituent and a second  $\alpha$  substituent selected from methyl and halogen, the absolute configuration is the same as that of D-glyceraldehyde with the second  $\alpha$  substituent taking the place of the hydrogen atom at the glyceraldehyde  $\alpha$ -carbon.

The peptides, portions of the peptides, variations/derivatives of the peptides or portions of the variations/derivatives described herein can be used as inhibitors of HIV entry into cells. The peptides represented in FIG. 3 or a portion of a peptide sufficient to fit into the hydrophobic pocket at the C-terminal end of the coiled-coil and prevent interaction of the C-peptide region with the N-peptide region of gp41 are useful to inhibit HIV infection. A portion of any of the peptides represented or of a derivative thereof can be from 2 to 20 (any number of residues from 2 to 20) amino acid residues in size. D-peptides which comprise the consensus sequence tryptophan-tryptophan-leucine or the sequence tryptophan-tryptophan-leucine-glutamate, described herein, and additional residues, can be used; the other residues present in such D-peptides and the size of the D-peptides can be selected with reference to peptides described herein or can be designed independent of those peptides, provided that these three or four residues are positioned in such a manner that the peptide can fit into the hydrophobic pocket and act as an inhibitor. Additional amino acid residues can also be present at the N-terminus, the C-terminus or both of the D-peptides described herein, thus producing a larger peptide. Alternatively, there can be other amino acid residues selected, for example, to enhance binding affinity. Alternatively, a peptide which comprises the conserved amino acid residues of the D-peptides of FIG. 3 can be used. For example, such a peptide can be 16 amino acid residues in size and include the conserved amino acid residues, which can be at the same positions as those at which they occur in the peptides shown in FIG. 3. The intervening amino acid residues can be different from the amino acid residues at these positions in any of the peptides shown in FIG. 3 (e.g., can be isoleucine or asparagine or other amino acid residue which does not appear in the peptides represented in FIG. 3) or can be substituted for or replaced by an amino acid residue represented at a specific

position in another peptide shown in FIG. 3 (e.g., the aspartic acid residue in D10pep1 can be replaced by a serine residue). Amino acid residues other than the D-versions of the 20 L-amino acids found in natural proteins can be used. Such changes can be made, for example, to enhance bioavailability, binding affinity or other characteristic of the peptide. A D-peptide can comprise the conserved amino acid residues present in the peptides shown in FIG. 3, but they can be separated by fewer (or more) amino acid residues than the number of intervening amino acid residues shown in FIG. 3. For example, fewer than five amino acid residues (e.g., Tarrago-Litvak, L. et al., *FASEB J.*, 8:497 (1994); Tucker, T. J. et al., *Methods Enzymol.*, 275:440 (1996); Tarrago-Litvak, L. et al., *FASEB J.*, 8:497 (1994); Tucker, T. J. et al., *Methods Enzymol.*, 275:440 (1996)), can be present between the first cysteine and the glutamic acid in the consensus sequence shown in FIG. 3. Alternatively, these two residues can be separated by more than five amino acid residues. Internal modifications can also be made (e.g., to enhance binding or increase solubility of a peptide). For example, the first tryptophan of D10pep5 can be replaced by an arginine to increase solubility. A D-peptide can have additional moieties or amino acids at its N-terminus. For example, a moiety which blocks the N terminus or gets rid of the charge otherwise present at the N-terminus can be added. The moiety can be, for example, a blocking moiety, such as an acetyl group linked directly to the glycine (G), or an acetyl group linked to one or more additional amino acid residues linked to the N-terminal of G, such as an acetyl group linked to one or more lysine residues, which, in turn, are linked to the N terminal G. In one embodiment, two lysine residues are linked to the N-terminal G (KKGAC . . . ), for example to increase the solubility of the peptide; a blocking moiety, such as an acetyl group, can be linked to the terminal lysine (acetyl group KKGAC . . . ). In another embodiment, four lysine residues are linked to the N-terminal G. In addition, a D-peptide can have additional and/or altered moieties or amino acids at its C-terminus. For example, one or both of the alanine residues at the C-terminus can be altered and/or one or more residues can be added at the C-terminus, for example to enhance binding. Alternatively, functional (chemical) groups other than amino acid residues can be included to produce an inhibitor of the present invention. For example, these additional chemical groups can be present at the N-terminus, the C-terminus, both termini or internally. In addition, two or more D-peptides can be linked via an appropriate linker (e.g., a linker of amino acid residues or other chemical moieties) to increase the effectiveness of inhibition. Alternatively, one or more D-peptides can be linked via an appropriate linker to a molecule (drug) that binds to HIV gp120, CD4, CCR5, CXCR4, or a non-pocket region of HIV gp41 to increase the effectiveness of inhibition.

The D-peptides (or L-peptides or peptides with both D- and L-amino acids) can be produced using known methods, such as chemical methods or recombinant technology. The polypeptide backbone can be altered (e.g., N-methylation) or replaced with alternative scaffolds (e.g., peptoids) at one or more positions of the peptides. Additional components can be included in the peptides, such as, for example, linkers (chemical, amino acid) which are positioned between amino acids or amino acid portions of the peptide (e.g., to provide greater flexibility or to provide greater rigidity). As described herein, the D-peptides of the present invention are flanked by GA at the N-terminus and AA at the C-terminus, due to the design of the library used in identifying the D-peptides. Some or all of these four amino acid residues



may be altered, replaced or deleted in order to produce D-peptides with, for example, altered absorption, distribution, metabolism and/or excretion. In one embodiment, the C-terminus is modified by the addition of a glycine residue immediately before the C-terminal amide. In another embodiment, the most C-terminal A is altered/modified or replaced by a different amino acid residue or deleted.

D-peptides, which are of the opposite handedness from the handedness of naturally-occurring peptides, do not serve as efficient substrates for enzymes, such as proteases, and, therefore, are not as readily degraded as L-peptides. In addition, there is no effective immune response which targets D-peptides and therefore, they do not elicit an immune response comparable to that elicited by L amino acid peptides.

The present invention is illustrated by the following examples, which are not intended to be limiting in any way.

#### EXAMPLE 1

##### Synthesis of Variants of the C34 Peptide

Mutant peptides were synthesized by solid-phase Fmoc peptide chemistry and have an acetylated amino terminus and an amidated carboxy terminus. After cleavage from the resin, peptides were desalted with a Sephadex G-25 column (Pharmacia), and then purified by reverse-phase high-performance liquid chromatography (Waters, Inc.) on a Vydac C18 preparative column using a linear water-acetonitrile gradient and 0.1% trifluoroacetic acid. Peptide identities were verified by MALDI mass spectrometry (Voyager Elite, PerSeptive Biosystems). Peptide concentrations were measured by tryptophan and tyrosine absorbance in 6 M GuHCl [H. Edelhoch, *Biochemistry*, 6:1948 (1967)].

#### EXAMPLE 2

##### Quantitation of Helical Content and Thermal Stability of Mutant N36/C34 Complexes

CD measurements were performed in phosphate-buffered saline (50 mM sodium phosphate, 150 mM NaCl, pH 7.0) with an Aviv Model 62DS spectrometer as previously described (M. Lu, S. C. Blacklow, P. S. Kim, *Nat. Struct. Biol.*, 2:1075 (1995)). The apparent melting temperature of each complex was estimated from the maximum of the first derivative of  $[\theta]_{222}$  with respect to temperature. The mean residue ellipticities  $[\theta]_{222}$  ( $10^3 \text{ deg cm}^2 \text{ dmol}^{-1}$ ) at 0° C. were as follows: wildtype, -31.7; Met<sup>629</sup>→Ala, -32.0; Arg<sup>633</sup>→Ala, -30.7; Ile<sup>635</sup>→Ala, -25.9; Trp<sup>628</sup>→Ala, -27.0; Trp<sup>631</sup>→Ala, -24.9. In the case of the Trp<sup>628</sup>→Ala and Trp<sup>631</sup>→Ala mutations, the decrease in  $[\theta]_{222}$  is likely to overestimate the actual reduction in helical content. The removal of tryptophan residues from model helices has been reported to significantly reduce the absolute value of  $[\theta]_{222}$  even when there is little change in helical content (A. Chakrabarty, T. Kortemme, S. Padmanabhan, R. L. Baldwin, *Biochemistry*, 32:5560 (1993)).

#### EXAMPLE 3

##### Identification of Peptides Which Bind to a Pocket on the Surface of the N-helix Coiled-Coil of HIV-1 gp41.

Methods are available to identify D-peptides which bind to a cavity on the surface of the N-helix coiled-coil of HIV envelope glycoprotein gp41. As described in detail below,

D-peptides which bind to a cavity on the surface of the N-helix coiled-coil of HIV-1 envelope glycoprotein gp41 were identified by mirror-image phage display. This method involves the identification of ligands composed of D-amino acids by screening a phage display library. D-amino acid containing ligands have a chiral specificity for substrates and inhibitors that is the opposite of that of the naturally occurring L-amino ligands. The phage display library has been used to identify D-amino acid peptide ligands which bind a target or desired L-amino acid peptide (Schumacher et al. *Science*, 271:1854-1857 (1996)).

D-peptides that bind to the hydrophobic pocket of gp41 were identified using a target that is an enantiomer of IQN17, a hybrid molecule containing 29 residues of GCN4-pI<sub>2</sub>I on the N-terminal end and 17 residues of gp41 on the C-terminus. The phage library used for selection is described in U.S. Pat. No. 5,780,221 and Schumacher et al. *Science*, 271:1854-1857 (1996). The complexity of the library is greater than  $10^8$  different sequences. The sequences are flanked on either end by either a cysteine or a serine, with ten random residues in the middle. These sequences are located in the pIII gene of the phage, a coat protein that is expressed as approximately five copies on the outer surface of the phage.

The following experimental procedures were used in the examples described herein.

##### Phage Display

Neutravidin (Pierce, 10  $\mu\text{g}$  in 100  $\mu\text{L}$  of 100 mM  $\text{NaHCO}_3$ ) was added to individual wells of a 96-well high-binding styrene plate (Costar) and incubated overnight on a rocking platform at 4° C. The neutravidin was removed and the wells were washed four times with a TBS/Tween solution. Biotinylated D-IQN17 (100  $\mu\text{L}$  of a 10  $\mu\text{L}$  peptide solution in 100 mM  $\text{NaHCO}_3$ ) was added to the wells and incubated for one hour at 25° C. The biotinylated target was removed and a blocking solution (30 mg/ml nonfat dried milk in 100 mM  $\text{NaHCO}_3$ ) was added to the wells and incubated for two hours, with rocking, at 4° C. The blocking solution was removed and the wells were coated again with the biotinylated target as above. The target was removed and the unliganded neutravidin was blocked by the addition of the blocking solution with 5 mM biotin. After removing the biotin, the wells were washed six times with the TBS/Tween solution. The phage stock was then added to the wells (50  $\mu\text{L}$  of phage stock plus 50  $\mu\text{L}$  of phage-binding buffer: TBS, 0.1% Tween-20, 1 mg/ml milk, 0.05% sodium azide). The incubation time of the phage stock in the wells decreased in increasing rounds of selection. After incubation, the phage solution was removed and the wells were washed twelve times with TBS/Tween to remove the unbound phage. Odd numbered washes were performed quickly, with no incubation time; even numbered washes were incubated for increasing amounts of time each round of phage selection. The phage were eluted by the addition of two micrograms of trypsin in 100  $\mu\text{L}$  of phage-binding buffer and 2.5 mM  $\text{CaCl}_2$  with an hour incubation at 37° C. To determine recovery, a dilution of the eluted phage was used to infect K 91 kan cells. After a one hour incubation, 100  $\mu\text{L}$  of cells were removed and 1:10, 1:100, and 1:100 dilutions in LB were plated on LB/tetracycline plates. Phage recovery was determined as a ratio of transducing units recovered (the titer of the eluted phage) to the input number of transducing units (the titer of the phage stock used that round). Transducing units were determined by counting the number of tetracycline-resistant colonies on the LB/tetracycline plates. Non-specific phage recovery generally has a ratio in the order of magnitude of  $10^{-8}$  to  $10^{-9}$ , whereas specifically

amplified phage have a ratio  $10^{-7}$  or greater. Individual clones were amplified and sequenced. They were assayed in the binding assay to determine binding specificity.

D10pep7 was identified after five rounds of phage selection. D10pep1, D10pep3, D10pep4, D10pep5, and D10pep6 were identified after seven rounds of phage selection. The phage selection was performed again, with shorter incubation times and longer washes, and D10pep10 and D10pep12 were identified after three rounds of selection. (A ninth D-peptide was identified but was not further investigated once it was shown to be toxic to cells.)

To test the specificity of binding of identified phage clones to the pocket of D-IQN17, the phage clones were added to wells of 96-well plates coated as above with D-IQN17, D-GCN4-pI<sub>Q</sub>I (with the three mutations), D-IQN17(G39W=glycine36 substituted with tryptophan), or wells with no target. The phage were incubated on the plates and washed for the same lengths of time as in the round from which they were identified. Eluted phage were used to infect K91 kan cells and the recovered transducing units were determined as above. These sequences bound specifically to the wells with D-IQN17.

#### Peptide Purification

IQN17 and the D10 peptides were synthesized by Fmoc peptide chemistry. They have an acetylated N-terminus and a C-terminal amide. IQN17 contains 29 residues derived from GCN4-pI<sub>Q</sub>I on the N-terminus and 17 residues from the C-terminus of N36 on the C-terminus. There is one residue overlap between GCN4-pI<sub>Q</sub>I and the N36 region, making the peptide 45 residues long. To improve solubility, three amino-acid substitutions were made in the GCN4-pI<sub>Q</sub>I region of IQN17, as compared to the original GCN4-pI<sub>Q</sub>I sequence (Eckert, D. M. et al., *J. Mol. Biol.*, 284:859-865 1998). These substitutions are L13E, Y17K, and H18K. Thus, the sequence of IQN17 is: ac-RMKQIEDKIEEIESKQKKIENEIARIKLLQLTVWGIKQLQARIL-am (ac- represents an N-terminal acetyl group and -am represents a C-terminal amide), with the HIV portion underlined. For mirror-image phage display, IQN17 was synthesized using D-amino acids (for amino acid residues that contain a second chiral center, such as Ile and Thr, the exact mirror image of the naturally occurring amino acid residue is used to create the D-version of the target). In addition, the N-terminus of the peptide was biotinylated using NHS-LC-biotin-II (Pierce, catalog #21336). Between the biotin and the IQN17 sequence was a three amino acid linker of GKG, with the lysine in the naturally-occurring L-form. This lysine was inserted as a trypsin recognition site.

The sequences of the D-peptides are as follows (with all amino acids in the D-enantiomer, using the exact mirror image of naturally occurring amino acid residues for Ile and Thr, which contain a second chiral center):

D10pep1: Ac-GACEARHREWAWLCAA-CONH<sub>2</sub> (SEQ ID NO: 34);

D10pep3: Ac-KKGACGLGQEEFWLCAA-CONH<sub>2</sub> (SEQ ID NO: 64);

D10pep4: Ac-GACDLKAKEFWLCAA-CONH<sub>2</sub> (SEQ ID NO: 35);

D10pep5: Ac-KKGACCELLGWEWAWLCAA-CONH<sub>2</sub> (SEQ ID NO: 65);

D10pep6: Ac-GACSRSQPEWEWLCAA-CONH<sub>2</sub> (SEQ ID NO: 36);

D10pep7: Ac-GACLLRAPEWGWLCAA-CONH<sub>2</sub> (SEQ ID NO: 37);

D10pep10: Ac-KKGACMRGEWWSWLCAA-CONH<sub>2</sub> (SEQ ID NO: 67); and

D10pep12: Ac-KKGACPLNKEWAWLCAA-CONH<sub>2</sub> (SEQ ID NO: 68).

After cleavage from the resin, the peptides were desalted on a Sephadex G-25 column (Pharmacia) and lyophilized. The lyophilized peptides were purified by reverse-phase high performance liquid chromatography (Waters, Inc.) on a Vydac C18 preparative column. The D-peptides were then air-oxidized by dissolving the lyophilized powder in 20 mM Tris, pH 8.2, and stirring at room temperature for several days. The oxidized peptides were HPLC purified as before. The expected molecular weights of the peptides were verified using MALDI-TOF mass spectrometry (PerSeptive Biosystems). Peptide concentrations were determined using tyrosine, tryptophan and cysteine absorbance at 280 nm in six molar GuHCl (Edelhoch, 1967). Peptide stock solutions were prepared in DMSO.

The N-terminal lysines on D10pep3, D10pep5, D10pep7a, D10pep10 and D10pep12 were added to increase the water solubility of the peptides. To investigate the effect of the added lysines on the inhibitory activity of the peptides, D10pep1 was synthesized with two N-terminal lysines (denoted D10pep1a) and compared to D10pep1 without lysines: D10pep1a was found to have an IC<sub>50</sub> for inhibition of syncytia formation approximately 2-fold higher than D10pep1 (i.e., without lysines). In addition, D10pep5 was synthesized with two additional N-terminal lysines (for a total of four lysines to generate a peptide denoted D10pep5a). The IC<sub>50</sub> for inhibition of syncytia formation of D10pep5a was approximately 2-fold higher than D10pep5. The addition of N-terminal lysine residues to the D-peptides results in only a modest decrease of inhibitory activity.

D-peptides that had additional D-Lys residues added to the N-termini, that were synthesized for study are indicated with the addition of "a" to the peptide name and include the following:

D10pep1a: Ac-KKGACEARREWAWLCAA-CONH<sub>2</sub> (SEQ ID NO: 38);

D10pep4a: Ac-KKGACDLKAKEFWLCAA-CONH<sub>2</sub> (SEQ ID NO: 39);

D10pep5a: Ac-KKKKGACCELLGWEWAWLCAA-CONH<sub>2</sub> (SEQ ID NO: 66);

D10pep6a: Ac-KKGACSRSQPEWEWLCAA-CONH<sub>2</sub> (SEQ ID NO: 40); and

D10pep7a: Ac-KKGACLLRAPEWGWLCAA-CONH<sub>2</sub> (SEQ ID NO: 41).

These sequences are also represented in FIG. 3. The 12 amino acid "core" of each D-peptide (which, in turn comprises a 10-mer and the consensus sequences described herein) are as follows:

CDLKAKEFWLWC (SEQ ID NO: 3)

CEARHREWAWLWC (SEQ ID NO: 4)

CELLGWEWAWLWC (SEQ ID NO: 5)

CLLRAPEWGWLWC (SEQ ID NO: 6)

CSRSQPEWEWLC (SEQ ID NO: 7)

CGLGQEEFWLWC (SEQ ID NO: 8)

CMRGEWWSWLWC (SEQ ID NO: 9)

CPLNKEWAWLWC (SEQ ID NO: 10)

CVLKAKEFWLWC is an alternative sequence for peptide SEQ ID NO: 3. (SEQ ID NO: 11).

It is readily apparent that there is a highly conserved consensus sequence in these peptides. The 12 amino acid peptide represented in FIG. 3 can be represented as: CXXXXXEWXWLC (SEQ ID NO: 12), where amino acid residues common to the peptides are shown and X represents an amino acid residue which is not conserved among the peptides.

#### EXAMPLE 4

##### Assessment of Activity of C34 Peptides and D-Peptides

The potency of C34 peptides in inhibiting viral infection and the HIV-1 infection inhibitory activity of the D-peptides

were assayed using recombinant luciferase-expressing HIV-1 (Chen, B. K. et al., *J. Virol.*, 68:654 (1994); Malashkevich, V. N., et al. *Proc. Natl. Acad. Sci., USA*, 95:9134 (1998)). The virus was produced by co-transfecting an envelope-deficient HIV genome NL43LucR-E- (Chen, B. K. et al., *J. Virol.*, 68:654 (1994) and the HXB2 gp160 expression vector pCMVHXB2gp160 (see Chan, D. C. et al., *Proc. Natl. Acad. Sci.*, 95:11513 (1998)) into 293T cells. Low-speed centrifugation was used to clear the viral supernatants of cellular debris. The supernatant was used to infect HOS-CD4/Fusion cells (N. Landau, NIH AIDS Reagent Program) in the presence of the D-peptides, with concentrations ranging from 0 to 500  $\mu$ M. Cells were harvested 48 hours post-infection, and luciferase activity was monitored in a Wallac AutoLumat LB953 luminometer (Gaithersburg, Md.). The  $IC_{50}$  is the peptide concentration that results in a 50% decrease in activity relative to control samples lacking peptide. The  $IC_{50}$  was calculated from fitting the data to a Langmuir equation  $[y=k/(1+([peptide]/IC_{50})+x)]$ , where  $y$ =luciferase activity and  $k$  and  $x$  are scaling constants.

#### Cell/Cell Fusion Assay

Inhibition of cell/cell fusion (i.e., syncytia formation) was assayed by co-culturing Chinese hamster ovary cell expressing HXB2 envelope (K. Kozarsky, et al., *J. Acquir. Immune. Defic. Syndr.*, 2:163 (1989) and the HeLa-CD4-LTR-Beta-gal cells (M. Emerman, NIH AIDS Reagent program) in the presence of varying concentration of peptide. When mixed, these cells form syncytia, or multi-nucleated cells, which express  $\beta$ -galactosidase. Approximately twenty hours after co-culturing the cells, the monolayers were stained with 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactoside to visualize the syncytia. The syncytia are visualized with a microscope and counted manually (a syncytia is scored as a fused cell containing three or more nuclei). The  $IC_{50}$  was calculated from fitting the data to a Langmuir equation  $[y=k/(1+[peptide]/IC_{50})+x]$ , where  $y$ =number of syncytia and  $k$  and  $x$  are scaling constants.

TABLE 1

Stability of mutant N36/C34 complexes and the inhibitory potency of C34 mutants.			
Peptide	$T_m$ ( $^{\circ}$ C.)	$IC_{50}$ (nM) viral entry	$IC_{50}$ (nM) cell fusion
Wildtype Cavity-binding	66	$2.1 \pm 0.31$	$0.55 \pm 0.03$
Trp <sup>628</sup> $\rightarrow$ Ala	53	$10 \pm 2.0$	$3.8 \pm 0.33$
Trp <sup>631</sup> $\rightarrow$ Ala	37	$61 \pm 16$	$15 \pm 0.82$
Ile <sup>635</sup> $\rightarrow$ Ala	55	$4.1 \pm 0.91$	$0.96 \pm 0.12$
Control residues			
Met <sup>620</sup> $\rightarrow$ Ala	66	$2.0 \pm 0.27$	$0.74 \pm 0.03$
Arg <sup>633</sup> $\rightarrow$ Ala	65	$2.6 \pm 0.89$	$0.76 \pm 0.07$

Mutant C34 peptides (10  $\mu$ M) were complexed with the N36 peptide (10  $\mu$ M) in phosphate-buffered saline (pH 7.0) for circular dichroism (CD) measurements. The apparent melting temperatures ( $T_m$ ) were estimated from the thermal dependence of the CD signal at 222 nm. Inhibition of viral entry was measured in a cell-culture infection assay using recombinant luciferase-expressing HIV-1. Inhibition of cell-cell fusion was measured in a syncytium assay. The means and standard errors are from triplicate trials.

Similarly, the activity of the D-peptides described was assessed using the two assays described above. Results are shown in FIGS. 6A-6B and 8A-8B.

## EXAMPLE 5

#### Crystallization of the IQN17/D10pep1 Complex and Ligand-Free IQN17

##### Peptide Purification, Crystallization

Peptides IQN17 and D10pep1 were synthesized by Fmoc peptide chemistry, as described above.

A 10 mg/ml stock of a mixture of IQN17 and D10pep1 was prepared in water. The final concentration of IQN17 was about 1.37 nM, and the final concentration of D10pep1 was about 1.51 mM. Initial crystallization conditions were found using Crystal Kits I and II (Hampton Research), and then optimized. To grow the best diffracting crystals, one microliter of this stock was added to one microliter of the reservoir buffer (10% PEG 4000, 0.1 M NaCl pH 5.6, 20% 2-propanol) and allowed to equilibrate against the reservoir buffer. Crystals belong to a space group P321 ( $a=b=41.83$  Å;  $c=84.82$  Å,  $\alpha=\beta=90^{\circ}$ ,  $\gamma=120^{\circ}$ ) and contain one IQN17/D10pep1 monomer in the asymmetric unit. A useful osmium derivative was produced by increasing the concentration of PEG 4000 in the reservoir solution by 4%, adding  $(NH_4)_2OsCl_6$  to the reservoir solution to a final concentration of 5 mM and adding five microliters of the resulting solution to the drop containing the protein crystal. Prior to data collection native and heavy-atom derivative crystals were transferred into cryosolution containing 20% PEG 4000, 0.1 M NaCl pH 5.6, 20% 2-Propanol and flash-frozen using X-stream cryogenic crystal cooler (Molecular Structure Corporation).

The best diffracting crystals of ligand-free IQN17 were grown with a similar technique as above: on microliter of a 10 mg/ml solution of IQN17 in water was added to one microliter of the reservoir buffer (1.0 M  $K_2Na$  Tartrate, 0.1 M NaHEPES pH 7.0) and allowed to equilibrate against the reservoir buffer. Before flash freezing, the crystals were transferred into buffers consisting of the reservoir solution with increasing amounts of glycerol, up to a final concentration of 23% glycerol. Crystals belong to the space group C222<sub>1</sub> ( $a=57.94$  Å,  $b=121.96$  Å,  $c=73.67$  Å;  $\alpha=\beta=\gamma=90^{\circ}$ ) and contain one IQN17 trimer in the asymmetric unit.

##### X-Ray Data Collection and Processing

Initial data were collected on a Rigaku RU300 rotating-anode x-ray generator mounted to an R-axis IV area detector (Molecular Structure Corporation). Diffraction data for IQN17 were collected at 100 K using a Quantum-4 CCD detector and the 5.0.2 beamline at the Advanced Light Source (Berkeley, USA). Final native and multiwavelength anomalous diffraction (MAD) data for IQN17/D10pep1 were collected at the Howard Hughes Medical Institute Beamline X4A at Brookhaven National Laboratory using a Raxis-IV detector. For MAD data, four wavelengths near the osmium L-III absorption edge were selected based on the fluorescence spectrum of the Os derivative crystal (Table 2). The four wavelengths were: 1.1398 Å, 1.1403 Å, 1.1393 Å, 1.1197 Å. Data sets were collected in 20° batches, allowing the same batch to be collected at each wavelength before moving to the next batch, in order to minimize the crystal decay between data sets. Reflections were integrated and scaled with the programs DENZO and SCALEPACK (Otwinowski, Z., (1993) in *Data Collection and Processing*, eds. Sawyer, L., Isaacs, N. & Bailey, S. (SERC, Daresbury Laboratory, Warrington, England), pp. 55-62).

Further diffraction data processing, phase determination and map calculations were performed using the CCP4 suite of programs (CCP4, *Acta Cryst. D*50:760-763 (1994)). Intensities were reduced to amplitudes with the program TRUNCATE, and the data sets for the wavelengths closest to the Os L-II absorption edge ( $\lambda_1, \lambda_2, \lambda_3$ ) were scaled with SCALEIT to the remote wavelength ( $\mu_4$ ) data set (Table 2).

## Phase Determination and Crystallographic Refinement

Initially, phase determination for IQN17/D10pep1 crystals was attempted with the molecular replacement technique using the theoretical model of IQN17 build from the published GCN4-pI<sub>2</sub>I and HIV gp41 structures (Eckert, D. M., et al. (1998) *J. Mol. Biol.* 284:859-865; Chan, D. C., et al. (1997) *Cell* 89, 263-273) with sidechains truncated to a polyserine chain. The resulting molecular replacement solutions were ambiguous and the electron density map did not reveal conformation of the D10pep1 peptide. The molecular replacement phases were good enough, however, for determining the coordinates of a single Os atom in the corresponding derivative using difference and anomalous fourier maps. The heavy atom binds on the crystallographic three-fold axis (0.333, 0.667, 0.047). MAD phases were then generated with the program MLPHARE (Table 2) and extended to higher resolution with the program DM. The quality of MAD electron density map at 1.5 Å resolution was exceptional, and revealed structural details of IQN17 and D10pep1 peptide with clarity. Electron density map interpretation and model building was done with the program O (Jones, T. A. et al. (1991) *Acta Crystallogr.* D47, 110-119). The structure of IQN17-D10pep1 complex was refined using the program CNS (Brünger, A. T. et al., *Acta Crystallogr.* D54, 905-921 (1998)). The correctness of the structure was checked with simulated annealing omit maps and with the program WHAT CHECK (Hoff, R. W. W. et al., *Nature* 381: 272 (1996)). All residues of IQN17 and the D10pep1 peptide (when converted into its mirror image) occupy most preferred areas of the Ramachandran plot. The conformations of the majority of the residues are well defined except for the two most N-terminal residues of IQN17 and the side chains of Arg-6 and Arg-8 of the D10pep1 peptide.

The structure of ligand-free IQN17 was solved by molecular replacement using the program AMORE (Navaza, J. (1994) *Acta Crystallogr.* A50, 157-163) and the IQN17 part of the refined IQN17/D10pep1 structure as a test model. Three-fold noncrystallographic averaging, solvent flattening and histogram matching with the program DM was used for phase improvement. Electron density map interpretation and model building was done with the program O (Jones et al., *Acta Crystallogr.* D54, 905-921 (1991)). The structure of the IQN17/D10pep1 complex was refined using the program CNS (Brünger, A. T. et al., *Acta Crystallogr.* D54, 905-921 (1998)).

The crystal structure can be used to design more effective and/or new D-peptides, peptidomimetics or other small molecules that inhibit HIV infectivity.

## EXAMPLE 6

## Nuclear Magnetic Resonance (NMR) Methods for Identifying Compounds Which Bind to the N-Helix Hydrophobic Pocket of gp41

## A. Assaying Specific Binding Between the IQN17 Hydrophobic Pocket and D-Peptides

NMR experiments were used to assay the binding of each D-peptide to IQN17. The single tryptophan residue of IQN17 (denoted Trp-571) provides an excellent probe of specific binding to the hydrophobic pocket of gp41. In deuterium oxide (deuterated water) buffers, the simple homonuclear one-dimensional <sup>1</sup>H NMR spectrum of IQN17 (FIG. 9A, middle) shows five signals from the Trp-571 indole, extremely well-resolved from all other signals in the molecule. To test a compound for binding to the gp41 pocket, two one-dimensional <sup>1</sup>H NMR measurements were made on samples in deuterated buffers. First, a reference (control) spectrum of IQN17 was taken, identifying the

Trp571 chemical shifts in the unbound form. A second spectrum was acquired on a sample containing both IQN17 and the compound in question. An optional third spectrum of the D-peptide (or other small molecule, or mix of molecules) was also taken. <sup>1</sup>H NMR experiments were performed on a Bruker AMX 500 spectrometer. Data was processed in Felix 98.0 (MSI) on Silicon Graphics computers, and all spectra were referenced to DSS. All experiments were performed at 25° C. in 100 mM NaCl, 50 mM sodium phosphate (pH 7.5). All buffers used were >99.7% D<sub>2</sub>O, to remove overlapping resonances from exchangeable backbone and side chain protons. Solute concentrations ranged from 0.3-1.0 mM for individual peptides, 0.8-1.0 mM for 1:1 complexes of IQN17 with each D-peptide.

Simple binding of two or more components is expected to result both in broader peaks (due to the increased size of the complex) and in changes in chemical shifts (due to the different chemical environments experienced by nuclei in free and bound forms). Specific binding to the hydrophobic pocket is indicated by a change in the Trp-571 chemical shifts, as well as by a broadening of peaks. Binding can also be indicated by similar changes in the chemical shifts and peak widths of the molecule (peptides and small organic molecules, for example) assayed. FIG. 9A shows an example of these effects: the NMR spectrum of the IQN17/D10pep1a complex displays broader peaks and dramatically different chemical shifts than the spectra for either of the two separate components. All IQN17/D-peptide complexes studied gave similar results, though varying in the degree of chemical shift dispersion (FIG. 9B). Thus, binding was indicated in all cases.

The x-ray crystallographic finding that the two conserved Trp residues, and the conserved Leu residue, in D10pep1 are directly involved in the binding of the IQN17 pocket, strongly suggests that these conserved residues participate in a similar manner when the other D-peptides bind the pocket. These conserved tryptophan residues, and Trp-571 of IQN17, provide an opportunity to study the binding interfaces in greater detail. In the IQN17/D10pep1 crystal structure, the Trp-571 sidechain of IQN17 is in close contact with Trp-10 of D10pep1, with several protons of Trp-571 (H<sub>ε2</sub>, H<sub>η2</sub>, H<sub>ε3</sub>, H<sub>ε3</sub>; the four scalar-coupled protons of the aromatic ring) above the plane of the Trp-10 indole group. In this position, aromatic ring current interactions (F. A. Bovey, *Nuclear Magnetic Resonance Spectroscopy* (1988)) are expected to alter the chemical shifts of some of those protons, moving peaks upfield in the manner seen (FIG. 9A, bottom). Use of the structure-based chemical shift prediction program SHIFTS (version 3.0b2, K. Osapay, D. Sitkoff, D. Case) also predicted that only protons from Trp-571 will experience a large upfield shift, especially the HC<sub>ε3</sub> proton. If the other D-peptides bind to the IQN17 pocket in the same fashion as D10pep1, a similar juxtaposition of Trp-571 and Trp-10 should occur, resulting in upfield-shifted peaks. All of the D-peptide/IQN17 complexes studied displayed such peaks, though varying in the extent of the shift (FIG. 9B). The D10pep1 complex showed the most extreme upfield shifts, and the D10pep7a complex the least. The magnitude of these changes is very large, ranging from roughly 0.5 to 2 ppm for the most upfield-shifted proton (H<sub>ε3</sub>, in all cases where it could be assigned). In comparison, chemical shift differences often used to detect binding in SAR by NMR experiments (Shuker, S. B., Hajduk, P. J., Meadows, R. P., Fesik, S. W., *Science* 274:1531-1534 (1996)) are frequently in the range of 0.05 to 0.2 ppm.) Though a broad range of upfield chemical shifts was observed, ring-current effects can be highly sensitive to distance and orientation, so that small

structural differences may give rise to substantial variations in chemical shift. (All of the upfield shifts observed are consistent with the approximate orientation of Trp side chains expected from the x-ray crystal structure.) Also, the upfield-shifted peaks are somewhat broadened compared to others in these NMR spectra (most likely due to some type of exchange process) an effect particularly pronounced for the complexes with D10pep5a and with D10pep7a.

To confirm that the strongly upfield-shifted peaks all correspond to a single sidechain (almost certainly Trp-571), two-dimensional NMR (TOCSY) experiments were performed on each of the IQN17/D-peptide complexes. As expected, the TOCSY experiments indicate that in each complex, the strongly upfield-shifted resonances all belong to the same aromatic side chain, identified as a group of four scalar-coupled protons. One example TOCSY spectrum is shown in FIG. 9C. For several of the complexes studied, NOESY experiments also indicate contact between this sidechain and other (unassigned) aromatic groups, as expected from the IQN17/D10pep1 structure. Not all of the potential NOE crosspeaks could be resolved, due to intense spectral overlap in the 6.8–7.6 ppm region. 2D NOESY and TOCSY experiments as described in J. Cavanaugh, W. J. Fairbrother, A. G. Palmer, N. J. Skelton, *Protein NMR Spectroscopy: Principles and Practice* (1996) were performed on samples of IQN17 and of each complex, with mixing times ranging between 30–90 ms (NOESY) and 30–70 ms (TOCSY). Spectral widths of 11,111 Hz and 5555 Hz were used in the acquisition ( $t_2$ ) and indirect ( $t_1$ ) dimensions, respectively. TOCSY experiments employed the DIPSI-2rc mixing sequence (J. Cavanaugh, M. Rance, *J. Magn. Reson. Ser. A*, 105:328 (1993)).

We conclude that all D-peptides assayed clearly bind the hydrophobic pocket of IQN17. Additionally, in the majority of these IQN17 complexes (i.e., D10pep1, D10pep3, D10pep4, D10pep6, D10pep10, and D10pep12) the D-peptides contact the pocket with very similar binding interfaces, bringing Trp-571 in close contact with the aromatic ring of Trp-10. In the cases of complexes with D10pep5a and D10pep7a this conclusion also seems very likely, although the more limited chemical shift dispersion and broader peaks raise a remote possibility of some other mode of binding.

The binding assay employed here can also be employed to assay binding of other molecules to the hydrophobic pocket of gp41 (e.g., such as found in IQN17). The assay is especially easy to interpret in a case where an aromatic group binds the pocket, as with the set of D-peptides described above. However, any pocket-binding molecules should also perturb the chemical shifts of Trp-571, an easily noticeable effect. In addition, new NMR signals generated by the small molecules themselves upon binding, are also indicative of binding.

The use of one-dimensional homonuclear  $^1\text{H}$  NMR provides significant advantages over multidimensional heteronuclear NMR to determine specific binding: (1) Sensitivity is higher, allowing samples to be assayed more quickly; alternately the higher sensitivity makes possible the use of lower concentrations of IQN17 and of putative binding agents, allowing screening for higher-affinity compounds, and more of them simultaneously. (2) Non-isotopically labeled proteins are simpler to produce, and more cost-effective. However, two-dimensional NMR experiments, either homonuclear or heteronuclear (with  $^{15}\text{N}$  and/or  $^{13}\text{C}$  isotopic labeling) could also be employed.

#### B. Screening chemical libraries

The binding assay described in (A) above can be used to screen large numbers of compounds present in a chemical library. Simple one-dimensional homonuclear  $^1\text{H}$  NMR experiments are sufficient to assess binding, with no requirement for isotopic labeling. Two-dimensional NMR experiments, either homonuclear or heteronuclear (with  $^{15}\text{N}$  and/or  $^{13}\text{C}$  isotopic labeling) could also be employed. Single compounds can be screened one at a time in this process. However, multiple compounds can also be combined in the same assay with IQN17 (or any representation of the gp41 N-helix coiled coil) and screened simultaneously. Binding to the pocket by any component of the mixture is indicated by a change in the Trp-571 chemical shifts. NMR signals from a large number of compounds together have the potential to obscure signals from Trp-571; these signals from unbound molecules can be eliminated using pulsed field gradient techniques well known in the art. With use of these techniques and a commercially available NMR tube sample changer, the automated screening of large numbers of compounds is straightforward.

#### C. Evaluating the products of multiple combinatorial syntheses

The screening process described in (B) above can also be extended to take advantage of combinatorial organic synthetic methods. Such methods are currently being used to generate whole families of compounds, with each family containing a diverse number of chemically related compounds. By the simple assay described above, the products of an entire combinatorial synthesis can be screened simultaneously. If no binding is indicated, then there is no need to invest further attention in any member of that family of compounds. If binding is indicated, then a particular family of promising compounds can be targeted for more detailed investigation. Simple one-dimensional homonuclear  $^1\text{H}$  NMR experiments are sufficient to assess binding, with no requirement for isotopic labeling. Two-dimensional NMR experiments, either homonuclear or heteronuclear (with  $^{15}\text{N}$  and/or  $^{13}\text{C}$  isotopic labeling) could also be employed.

TABLE 2

Data collection and refinement statistics				
Data collection				
Crystal	$\lambda$ (Å)	Completeness (%)	$R_{\text{sym}}^1$ (%)	Resolution (Å)
IQN17	1.0000	89.5	3.7	2.1
IQN17/D10	1.1197	93.8	4.8	1.5
Os $\lambda 1$	1.1403	98.6	6.3	2.0
Os $\lambda 2$	1.1399	96.8	9.7	2.0
Os $\lambda 3$	1.1393	96.9	7.9	2.0

TABLE 2-continued

Data collection and refinement statistics								
Os $\lambda 4$	1.1197	97.0		8.4		2.0		
<u>MAD phasing statistics (22.0–2.0 Å)</u>								
Derivative	$R_{\text{iso}}^2$ (%)	$R_{\text{outils}}^3$ Acentric	$R_{\text{outils}}^3$ Centric	$R_{\text{outils}}^3$ Anom.	Ph. Power <sup>4</sup> Acentric	Ph. Power <sup>4</sup> Centric	Occ. <sup>5</sup>	Anom. Occ. <sup>5</sup>
Os $\lambda 1$ vs. $\lambda 4$	7.3	0.75	0.61	0.47	1.41	1.21	−0.039	0.337
Os $\lambda 2$ vs. $\lambda 4$	5.2	0.83	0.71	0.44	1.04	1.15	−0.027	0.533
Os $\lambda 3$ vs. $\lambda 4$	3.3	0.97	0.97	0.49	0.35	0.28	−0.005	0.295
Overall figure of merit (before solvent flattening): 0.68								
<u>Refinement statistics</u>								
Crystal	Non-hydrogen protein atoms	Water s	Ions	Resolution (Å)	Reflections total	$R_{\text{cryst}}^6$	$R_{\text{free}}^6$	R.m.s. deviations bonds (Å) angles (°)
IQN17/D10	516	150	1	10.0–1.5	13549	0.214	0.245	0.012 1.498
IQN17	1143	160	1	5.0–2.5	7541	0.282	0.352	0.009 1.252

<sup>1</sup> $R_{\text{sym}} = \sum_j |I_j - \langle I \rangle| / \sum_j \langle I \rangle$ , where  $I_j$  is the recorded intensity of the reflection  $j$  and  $\langle I \rangle$  is the mean recorded intensity over multiple recordings.

<sup>2</sup> $R_{\text{iso}} = \sum |F_{\text{obs}} \pm F_{\text{calc}}| - |F_{\text{calc}}| / \sum |F_{\text{obs}}|$ , where  $F_{\text{obs}}$  is the structure factor at wavelength  $\lambda_i$  and  $F_{\text{calc}}$  is the structure factor at the reference wavelength  $\lambda_4$ .

<sup>3</sup> $R_{\text{outils}} = \sum |F_{\text{obs}} \pm F_{\text{calc}}| - |F_{\text{calc}}| / \sum |F_{\text{obs}}|$ , where  $F_{\text{calc}}$  is the calculated heavy atom structure factor.

<sup>4</sup>Phase power =  $\langle F_{\text{calc}} \rangle / E$ , where  $\langle F_{\text{calc}} \rangle$  is the root-mean-square heavy atom structure factor and  $E$  is the residual lack of closure error.

<sup>5</sup>Occupancies are values output from MLPHARE.

<sup>6</sup> $R_{\text{cryst, free}} = \sum |F_{\text{obs}}| - |F_{\text{calc}}| / |F_{\text{obs}}|$ , where the crystallographic and free R factors are calculated using the working and test sets, respectively. Test set contained 10% of reflections.

While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various

changes in form and details may be made therein without departing from the spirit and scope of the invention as defined by the appended claims.

## SEQUENCE LISTING

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Arg Ile Leu  
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Leu Cys

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 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Synthetic

<400> SEQUENCE: 26

Leu Leu Arg Leu Thr Val Trp Gly Thr Lys Asn Leu Gln Ala Arg Val  
 1 5 10 15

Thr

<210> SEQ ID NO 27  
 <211> LENGTH: 17  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Synthetic

<400> SEQUENCE: 27

Leu Leu Arg Leu Thr Val Trp Gly Thr Lys Asn Leu Gln Thr Arg Val  
 1 5 10 15

Thr

<210> SEQ ID NO 28  
 <211> LENGTH: 16  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Synthetic  
 <221> NAME/KEY: VARIANT  
 <222> LOCATION: (1)...(16)  
 <223> OTHER INFORMATION: Xaa = Any Amino Acid

<400> SEQUENCE: 28

Xaa Xaa Cys Xaa Xaa Xaa Xaa Xaa Glu Trp Xaa Trp Leu Cys Xaa Xaa  
 1 5 10 15

<210> SEQ ID NO 29  
 <211> LENGTH: 18  
 <212> TYPE: PRT

-continued

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```

<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic
<221> NAME/KEY: VARIANT
<222> LOCATION: (1)...(18)
<223> OTHER INFORMATION: Xaa - Any Amino Acid

<400> SEQUENCE: 29

```

```

Lys Lys Xaa Xaa Cys Xaa Xaa Xaa Xaa Xaa Glu Trp Xaa Trp Leu Cys
 1             5             10             15

```

```

Xaa Xaa

```

```

<210> SEQ ID NO 30
<211> LENGTH: 20
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic
<221> NAME/KEY: VARIANT
<222> LOCATION: (1)...(20)
<223> OTHER INFORMATION: Xaa - Any Amino Acid

```

```

<400> SEQUENCE: 30

```

```

Lys Lys Lys Lys Xaa Xaa Cys Xaa Xaa Xaa Xaa Xaa Glu Trp Xaa Trp
 1             5             10             15

```

```

Leu Cys Xaa Xaa
          20

```

```

<210> SEQ ID NO 31
<211> LENGTH: 17
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic
<221> NAME/KEY: VARIANT
<222> LOCATION: (1)...(17)
<223> OTHER INFORMATION: Xaa - Any Amino Acid

```

```

<400> SEQUENCE: 31

```

```

Xaa Xaa Cys Xaa Xaa Xaa Xaa Xaa Glu Trp Xaa Trp Leu Cys Xaa Xaa
 1             5             10             15

```

```

Xaa

```

```

<210> SEQ ID NO 32
<211> LENGTH: 19
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic
<221> NAME/KEY: VARIANT
<222> LOCATION: (1)...(19)
<223> OTHER INFORMATION: Xaa - Any Amino Acid

```

```

<400> SEQUENCE: 32

```

```

Lys Lys Xaa Xaa Cys Xaa Xaa Xaa Xaa Xaa Glu Trp Xaa Trp Leu Cys
 1             5             10             15

```

```

Xaa Xaa Xaa

```

```

<210> SEQ ID NO 33
<211> LENGTH: 21
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic
<221> NAME/KEY: VARIANT
<222> LOCATION: (1)...(21)
<223> OTHER INFORMATION: Xaa - Any Amino Acid

```

-continued

&lt;400&gt; SEQUENCE: 33

Lys Lys Lys Lys Xaa Xaa Cys Xaa Xaa Xaa Xaa Xaa Glu Trp Xaa Trp  
1 5 10 15  
Leu Cys Xaa Xaa Xaa  
20

&lt;210&gt; SEQ ID NO 34

&lt;211&gt; LENGTH: 16

&lt;212&gt; TYPE: PRT

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: Synthetic

&lt;400&gt; SEQUENCE: 34

Gly Ala Cys Glu Ala Arg His Arg Glu Trp Ala Trp Leu Cys Ala Ala  
1 5 10 15

&lt;210&gt; SEQ ID NO 35

&lt;211&gt; LENGTH: 16

&lt;212&gt; TYPE: PRT

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: Synthetic

&lt;400&gt; SEQUENCE: 35

Gly Ala Cys Asp Leu Lys Ala Lys Glu Trp Phe Trp Leu Cys Ala Ala  
1 5 10 15

&lt;210&gt; SEQ ID NO 36

&lt;211&gt; LENGTH: 16

&lt;212&gt; TYPE: PRT

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: Synthetic

&lt;400&gt; SEQUENCE: 36

Gly Ala Cys Ser Arg Ser Gln Pro Glu Trp Glu Trp Leu Cys Ala Ala  
1 5 10 15

&lt;210&gt; SEQ ID NO 37

&lt;211&gt; LENGTH: 16

&lt;212&gt; TYPE: PRT

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: Synthetic

&lt;400&gt; SEQUENCE: 37

Gly Ala Cys Leu Leu Arg Ala Pro Glu Trp Gly Trp Leu Cys Ala Ala  
1 5 10 15

&lt;210&gt; SEQ ID NO 38

&lt;211&gt; LENGTH: 18

&lt;212&gt; TYPE: PRT

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: Synthetic

&lt;400&gt; SEQUENCE: 38

Lys Lys Gly Ala Cys Glu Ala Arg His Arg Glu Trp Ala Trp Leu Cys  
1 5 10 15  
Ala Ala

&lt;210&gt; SEQ ID NO 39

&lt;211&gt; LENGTH: 18

-continued

<212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Synthetic  
 <400> SEQUENCE: 39

Lys Lys Gly Ala Cys Asp Leu Lys Ala Lys Glu Trp Phe Trp Leu Cys  
 1 5 10 15

Ala Ala

<210> SEQ ID NO 40  
 <211> LENGTH: 18  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Synthetic  
 <400> SEQUENCE: 40

Lys Lys Gly Ala Cys Ser Arg Ser Gln Pro Glu Trp Glu Trp Leu Cys  
 1 5 10 15

Ala Ala

<210> SEQ ID NO 41  
 <211> LENGTH: 18  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Synthetic  
 <400> SEQUENCE: 41

Lys Lys Gly Ala Cys Leu Leu Arg Ala Pro Glu Trp Gly Trp Leu Cys  
 1 5 10 15

Ala Ala

<210> SEQ ID NO 42  
 <211> LENGTH: 17  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Synthetic  
 <221> NAME/KEY: VARIANT  
 <222> LOCATION: (1)...(17)  
 <223> OTHER INFORMATION: Xaa = Any Amino Acid

&lt;400&gt; SEQUENCE: 42

Leu Leu Xaa Leu Thr Val Trp Gly Xaa Lys Xaa Leu Gln Xaa Arg Xaa  
 1 5 10 15

Xaa

<210> SEQ ID NO 43  
 <211> LENGTH: 20  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Synthetic

&lt;400&gt; SEQUENCE: 43

Lys Lys Lys Lys Gly Ala Cys Glu Ala Arg His Arg Glu Trp Ala Trp  
 1 5 10 15

Leu Cys Ala Ala  
 20

<210> SEQ ID NO 44  
 <211> LENGTH: 16

-continued

<212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Synthetic  
  
 <400> SEQUENCE: 44  
  
 Gly Ala Cys Gly Leu Gly Gln Glu Glu Trp Phe Trp Leu Cys Ala Ala  
 1 5 10 15

<210> SEQ ID NO 45  
 <211> LENGTH: 20  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Synthetic  
  
 <400> SEQUENCE: 45

Lys Lys Lys Lys Gly Ala Cys Gly Leu Gly Gln Glu Glu Trp Phe Trp  
 1 5 10 15  
  
 Leu Cys Ala Ala  
 20

<210> SEQ ID NO 46  
 <211> LENGTH: 20  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Synthetic  
  
 <400> SEQUENCE: 46

Lys Lys Lys Lys Gly Ala Cys Asp Leu Lys Ala Lys Glu Trp Phe Trp  
 1 5 10 15  
  
 Leu Cys Ala Ala  
 20

<210> SEQ ID NO 47  
 <211> LENGTH: 15  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Synthetic  
  
 <400> SEQUENCE: 47

Gly Ala Cys Glu Leu Leu Gly Trp Glu Trp Ala Trp Leu Cys Cys  
 1 5 10 15

<210> SEQ ID NO 48  
 <211> LENGTH: 20  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Synthetic  
  
 <400> SEQUENCE: 48

Lys Lys Lys Lys Gly Ala Cys Ser Arg Ser Gln Pro Glu Trp Glu Trp  
 1 5 10 15  
  
 Leu Cys Ala Ala  
 20

<210> SEQ ID NO 49  
 <211> LENGTH: 20  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Synthetic

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&lt;400&gt; SEQUENCE: 49

Lys Lys Lys Lys Gly Ala Cys Leu Leu Arg Ala Pro Glu Trp Gly Trp  
 1 5 10 15  
 Leu Cys Ala Ala  
 20

&lt;210&gt; SEQ ID NO 50

&lt;211&gt; LENGTH: 16

&lt;212&gt; TYPE: PRT

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: Synthetic

&lt;400&gt; SEQUENCE: 50

Gly Ala Cys Met Arg Gly Glu Trp Glu Trp Ser Trp Leu Cys Ala Ala  
 1 5 10 15

&lt;210&gt; SEQ ID NO 51

&lt;211&gt; LENGTH: 20

&lt;212&gt; TYPE: PRT

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: Synthetic

&lt;400&gt; SEQUENCE: 51

Lys Lys Lys Lys Gly Ala Cys Met Arg Gly Glu Trp Glu Trp Ser Trp  
 1 5 10 15  
 Leu Cys Ala Ala  
 20

&lt;210&gt; SEQ ID NO 52

&lt;211&gt; LENGTH: 16

&lt;212&gt; TYPE: PRT

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: Synthetic

&lt;400&gt; SEQUENCE: 52

Gly Ala Cys Pro Pro Leu Asn Lys Glu Trp Ala Trp Leu Cys Ala Ala  
 1 5 10 15

&lt;210&gt; SEQ ID NO 53

&lt;211&gt; LENGTH: 20

&lt;212&gt; TYPE: PRT

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: Synthetic

&lt;400&gt; SEQUENCE: 53

Lys Lys Lys Lys Gly Ala Cys Pro Pro Leu Asn Lys Glu Trp Ala Trp  
 1 5 10 15  
 Leu Cys Ala Ala  
 20

&lt;210&gt; SEQ ID NO 54

&lt;211&gt; LENGTH: 16

&lt;212&gt; TYPE: PRT

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: Synthetic

&lt;221&gt; NAME/KEY: VARIANT

&lt;222&gt; LOCATION: (1)...(16)

&lt;223&gt; OTHER INFORMATION: Xaa = Any Amino Acid

&lt;400&gt; SEQUENCE: 54



-continued

Gly Ala Cys Xaa Xaa Xaa Xaa Glu Trp Xaa Trp Leu Cys Ala Ala  
 1 5 10 15

<210> SEQ ID NO 55  
 <211> LENGTH: 18  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Synthetic  
 <221> NAME/KEY: VARIANT  
 <222> LOCATION: (1)...(18)  
 <223> OTHER INFORMATION: Xaa = Any Amino Acid

<400> SEQUENCE: 55

Lys Lys Gly Ala Cys Xaa Xaa Xaa Xaa Glu Trp Xaa Trp Leu Cys  
 1 5 10 15

Ala Ala

<210> SEQ ID NO 56  
 <211> LENGTH: 20  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Synthetic  
 <221> NAME/KEY: VARIANT  
 <222> LOCATION: (1)...(20)  
 <223> OTHER INFORMATION: Xaa = Any Amino Acid

<400> SEQUENCE: 56

Lys Lys Lys Lys Gly Ala Cys Xaa Xaa Xaa Xaa Glu Trp Xaa Trp  
 1 5 10 15

Leu Cys Ala Ala  
 20

<210> SEQ ID NO 57  
 <211> LENGTH: 16  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Synthetic  
 <221> NAME/KEY: VARIANT  
 <222> LOCATION: (1)...(16)  
 <223> OTHER INFORMATION: Xaa = Any Amino Acid

<400> SEQUENCE: 57

Xaa Xaa Cys Xaa Xaa Xaa Xaa Glu Trp Xaa Trp Leu Cys Xaa Xaa  
 1 5 10 15

<210> SEQ ID NO 58  
 <211> LENGTH: 18  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Synthetic  
 <221> NAME/KEY: VARIANT  
 <222> LOCATION: (1)...(18)  
 <223> OTHER INFORMATION: Xaa = Any Amino Acid

<400> SEQUENCE: 58

Lys Lys Xaa Xaa Cys Xaa Xaa Xaa Xaa Glu Trp Xaa Trp Leu Cys  
 1 5 10 15

Xaa Xaa

<210> SEQ ID NO 59  
 <211> LENGTH: 20  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence

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<220> FEATURE:  
 <223> OTHER INFORMATION: Synthetic  
 <221> NAME/KEY: VARIANT  
 <222> LOCATION: (1)...(20)  
 <223> OTHER INFORMATION: Xaa - Any Amino Acid  
 <400> SEQUENCE: 59  
 Lys Lys Lys Lys Xaa Xaa Cys Xaa Xaa Xaa Xaa Xaa Glu Trp Xaa Trp  
 1 5 10 15  
 Leu Cys Xaa Xaa  
 20

<210> SEQ ID NO 60  
 <211> LENGTH: 17  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Synthetic  
 <221> NAME/KEY: VARIANT  
 <222> LOCATION: (1)...(17)  
 <223> OTHER INFORMATION: Xaa - Any Amino Acid  
 <400> SEQUENCE: 60  
 Xaa Xaa Cys Xaa Xaa Xaa Xaa Xaa Glu Trp Xaa Trp Leu Cys Xaa Xaa  
 1 5 10 15  
 Xaa

<210> SEQ ID NO 61  
 <211> LENGTH: 19  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Synthetic  
 <221> NAME/KEY: VARIANT  
 <222> LOCATION: (1)...(19)  
 <223> OTHER INFORMATION: Xaa - Any Amino Acid  
 <400> SEQUENCE: 61  
 Lys Lys Xaa Xaa Cys Xaa Xaa Xaa Xaa Xaa Glu Trp Xaa Trp Leu Cys  
 1 5 10 15  
 Xaa Xaa Xaa

<210> SEQ ID NO 62  
 <211> LENGTH: 21  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Synthetic  
 <221> NAME/KEY: VARIANT  
 <222> LOCATION: (1)...(21)  
 <223> OTHER INFORMATION: Xaa - Any Amino Acid  
 <400> SEQUENCE: 62  
 Lys Lys Lys Lys Xaa Xaa Cys Xaa Xaa Xaa Xaa Xaa Glu Trp Xaa Trp  
 1 5 10 15  
 Leu Cys Xaa Xaa Xaa  
 20

<210> SEQ ID NO 63  
 <211> LENGTH: 12  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Synthetic  
 <221> NAME/KEY: VARIANT  
 <222> LOCATION: (1)...(12)  
 <223> OTHER INFORMATION: Xaa - Any Amino Acid

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<400> SEQUENCE: 63

Cys Xaa Xaa Xaa Xaa :  
1 5

<210> SEQ ID NO 64  
<211> LENGTH: 18  
<212> TYPE: PRT  
<213> ORGANISM: Artii  
<220> FEATURE:  
<223> OTHER INFORMAT:

<400> SEQUENCE: 64

Lys Lys Gly Ala Cys :  
1 5

Ala Ala

<210> SEQ ID NO 65  
<211> LENGTH: 18  
<212> TYPE: PRT  
<213> ORGANISM: Artii  
<220> FEATURE:  
<223> OTHER INFORMAT:

<400> SEQUENCE: 65

Lys Lys Gly Ala Cys :  
1 5

Ala Ala

<210> SEQ ID NO 66  
<211> LENGTH: 20  
<212> TYPE: PRT  
<213> ORGANISM: Artii  
<220> FEATURE:  
<223> OTHER INFORMAT:

<400> SEQUENCE: 66

Lys Lys Lys Lys Gly :  
1 5

Leu Cys Ala Ala  
20

<210> SEQ ID NO 67  
<211> LENGTH: 18  
<212> TYPE: PRT  
<213> ORGANISM: Artii  
<220> FEATURE:  
<223> OTHER INFORMAT:

<400> SEQUENCE: 67

Lys Lys Gly Ala Cys :  
1 5

Ala Ala

<210> SEQ ID NO 68  
<211> LENGTH: 18  
<212> TYPE: PRT  
<213> ORGANISM: Artii  
<220> FEATURE:  
<223> OTHER INFORMAT:

<400> SEQUENCE: 68

Lys Lys Gly Ala Cys :  
1 5

icial Sequence

ION: Synthetic

Ala Cys Glu Leu Leu Gly Trp Glu Trp Ala Trp  
10 15

icial Sequence

ION: Synthetic

Met Arg Gly Glu Trp Glu Trp Ser Trp Leu Cys  
10 15

icial Sequence

ION: Synthetic

Pro Pro Leu Asn Lys Glu Trp Ala Trp Leu Cys  
10 15

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-continued

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Xaa Glu Trp Xaa Trp Leu Cys  
10

icial Sequence

ION: Synthetic

Gly Leu Gly Gln Glu Glu Trp Phe Trp Leu Cys  
10 15

icial Sequence

ION: Synthetic

Glu Leu Leu Gly Trp Glu Trp Ala Trp Leu Cys  
10 15

-continued

Ala Ala

<210> SEQ ID NO 69  
 <211> LENGTH: 16  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: D-peptide  
 <221> NAME/KEY: VARIANT  
 <222> LOCATION: (0)...(0)  
 <223> OTHER INFORMATION: Xaa = 2, 3, 4, 5, 6, and 9  
 <221> NAME/KEY: MOD\_RES  
 <222> LOCATION: (0)...(0)  
 <221> NAME/KEY: AMIDATION  
 <222> LOCATION: (0)...(0)  
 <400> SEQUENCE: 69

Cys Xaa Xaa Xaa Xaa Xaa Glu Trp Xaa Trp Leu Cys Ala Ala Ala Met  
 1 5 10 15

<210> SEQ ID NO 70  
 <211> LENGTH: 6  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: D-peptide  
 <221> NAME/KEY: MOD\_RES  
 <222> LOCATION: (0)...(0)  
 <221> NAME/KEY: ACETYLATION  
 <222> LOCATION: (0)...(0)  
 <400> SEQUENCE: 70

Ala Cys Lys Lys Gly Ala  
 1 5

<210> SEQ ID NO 71  
 <211> LENGTH: 8  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: D-peptide  
 <221> NAME/KEY: MOD\_RES  
 <222> LOCATION: (0)...(0)  
 <221> NAME/KEY: ACETYLATION  
 <222> LOCATION: (0)...(0)  
 <400> SEQUENCE: 71

Ala Cys Lys Lys Lys Lys Gly Ala  
 1 5

What is claimed is:

1. A D-peptide consisting of amino acid sequence CDLKAKEWFWLC (SEQ ID NO: 3), wherein all amino acids are D-enantiomers.
2. A D-peptide which binds to the pocket of an N-helix coiled-coil of HIV gp41, wherein the peptide comprises the

50

amino acid sequence CDLKAKEWFWLC (SEQ ID NO: 3), and wherein all amino acids are D-enantiomers.

\* \* \* \* \*



US006107019A

**United States Patent** [19]

Allaway et al.

[11] Patent Number: **6,107,019**[45] Date of Patent: **Aug. 22, 2000****[54] METHOD FOR PREVENTING HIV-1 INFECTION OF CD4<sup>+</sup> CELLS**

[75] Inventors: **Graham P. Allaway**, Mohegan Lake; **Virginia M. Litwin**, Fayetteville; **Paul J. Maddon**, Elmsford; **William C. Olson**, Ossining, all of N.Y.

[73] Assignee: **Progenics Pharmaceuticals, Inc.**, Tarrytown, N.Y.

[21] Appl. No.: **08/876,078**

[22] Filed: **Jun. 13, 1997**

**Related U.S. Application Data**

[63] Continuation-in-part of application No. 08/831,823, Apr. 2, 1997.

[60] Provisional application No. 60/019,715, Jun. 14, 1996, and provisional application No. 60/014,532, Apr. 2, 1996.

[51] Int. Cl.<sup>7</sup> ..... **C12Q 1/70**

[52] U.S. Cl. .... **435/5; 435/7.2; 435/7.21; 435/7.24; 435/7.92; 435/7.93; 436/537; 436/542**

[58] Field of Search ..... **435/5, 7.2, 7.21, 435/7.24, 7.92, 7.93; 436/537, 542**

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Dragic, Tatjana, et al., "HIV-1 entry into CD4<sup>+</sup> cells is mediated by the chemokine receptor CC-CKR-5" *Nature* (Jun. 20, 1996) vol. 381:667-673 (Exhibit 6).

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(List continued on next page.)

*Primary Examiner*—Robert D. Budens

*Attorney, Agent, or Firm*—John P. White; Cooper & Dunham LLP

[57]

**ABSTRACT**

This invention provides methods for inhibiting fusion of HIV-1 to CD4<sup>+</sup> cells which comprise contacting CD4<sup>+</sup> cells with a non-chemokine agent capable of binding to a chemokine receptor in an amount and under conditions such that fusion of HIV-1 to the CD4<sup>+</sup> cells is inhibited. This invention also provides methods for inhibiting HIV-1 infection of CD4<sup>+</sup> cells which comprise contacting CD4<sup>+</sup> cells with a non-chemokine agent capable of binding to a chemokine receptor in an amount and under conditions such that fusion of HIV-1 to the CD4<sup>+</sup> cells is inhibited, thereby inhibiting the HIV-1 infection. This invention provides non-chemokine agents capable of binding to the chemokine receptor and inhibiting fusion of HIV-1 to CD4<sup>+</sup> cells. This invention also provides pharmaceutical compositions comprising an amount of the non-chemokine agent capable of binding to the chemokine receptor and inhibiting fusion of HIV-1 to CD4<sup>+</sup> cells effective to prevent fusion of HIV-1 to CD4<sup>+</sup> cells and a pharmaceutically acceptable carrier.

**12 Claims, 8 Drawing Sheets**

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Wu, Lijun, et al., "CCR5 Levels and Expression Pattern Correlate with Infectability by Macrophage-tropic HIV-1, In Vitro" *J. Exp. Med.* (May 5, 1997) vol. 185:1681-1691 (Exhibit 18).

Zhang, Y.J., et al., "Structure/Activity Analysis of Human Monocyte Chemoattractant Protein-1 (MCP-1) by Mutagenesis" *The Journal of Biological Chemistry* (Jun. 1994) vol. 269: 15918-15924 (Exhibit 19).

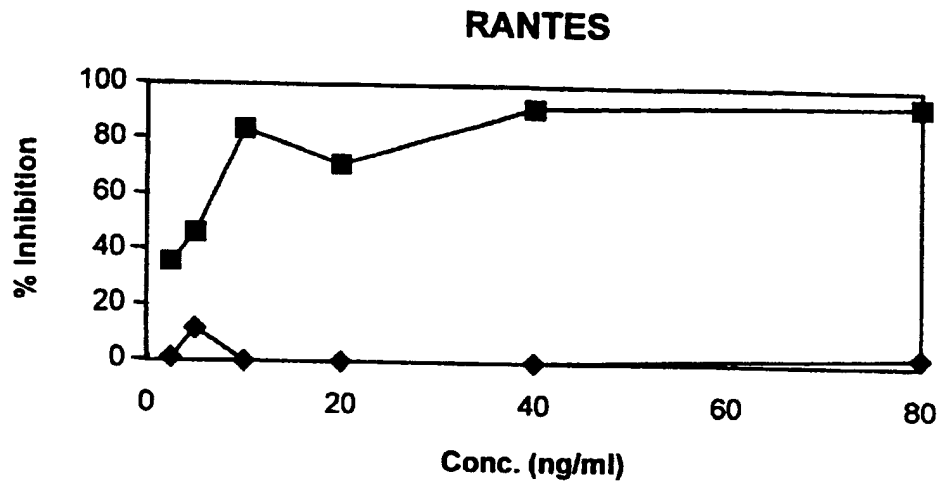
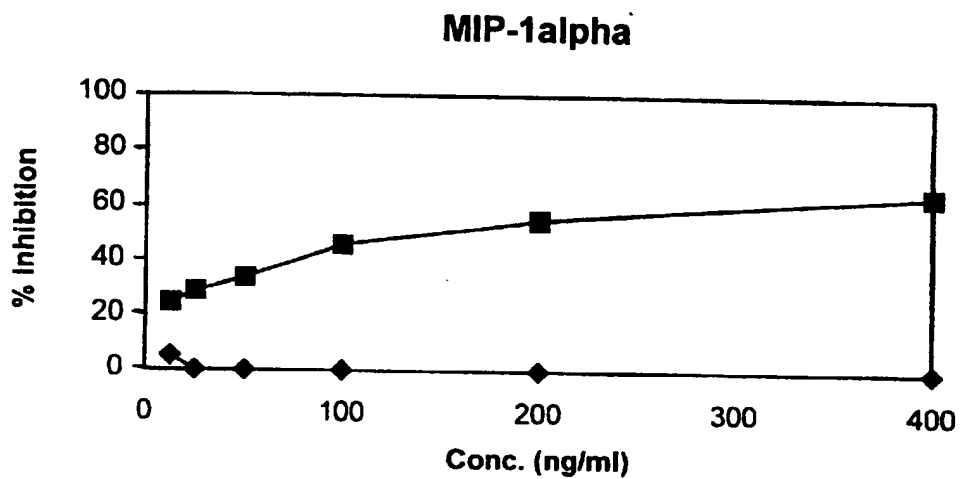
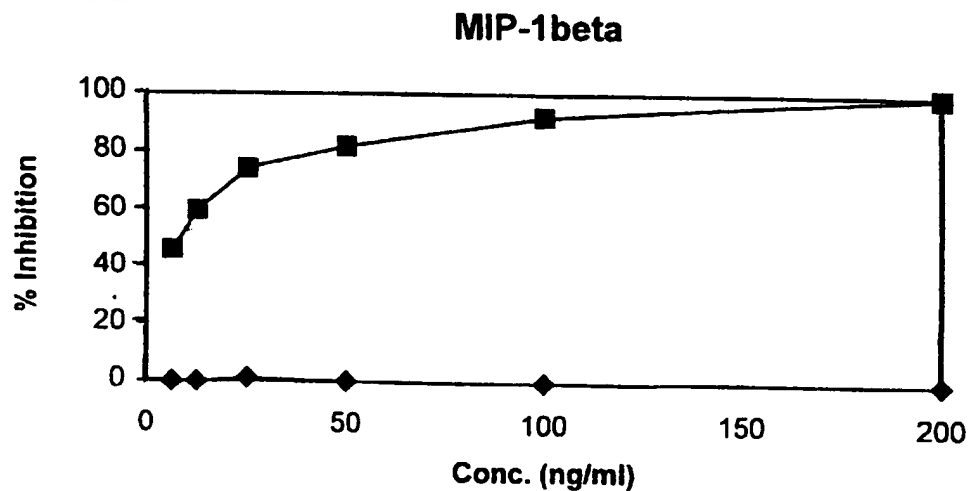
**FIG. 1A****FIG. 1B****FIG. 1C**



FIG. 2A

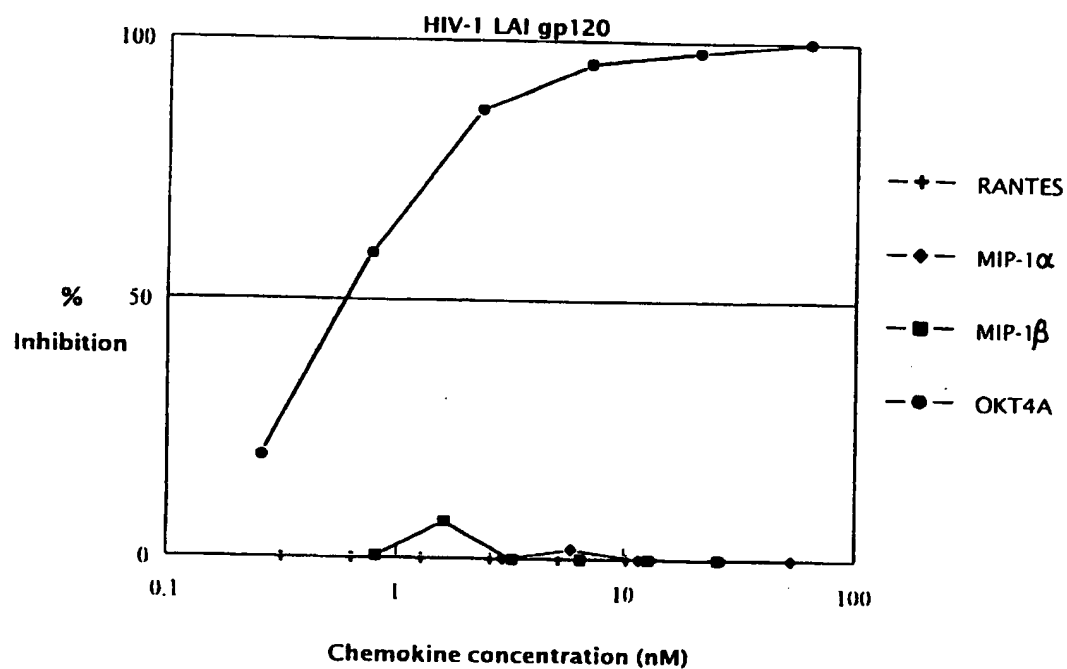


FIG. 2B

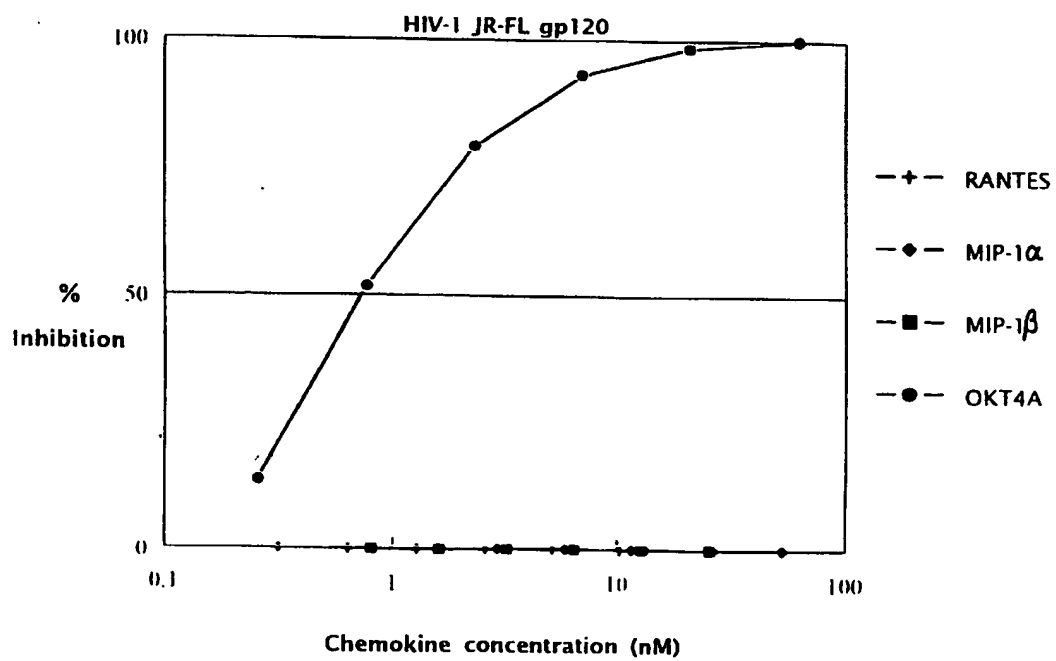


FIG. 3A

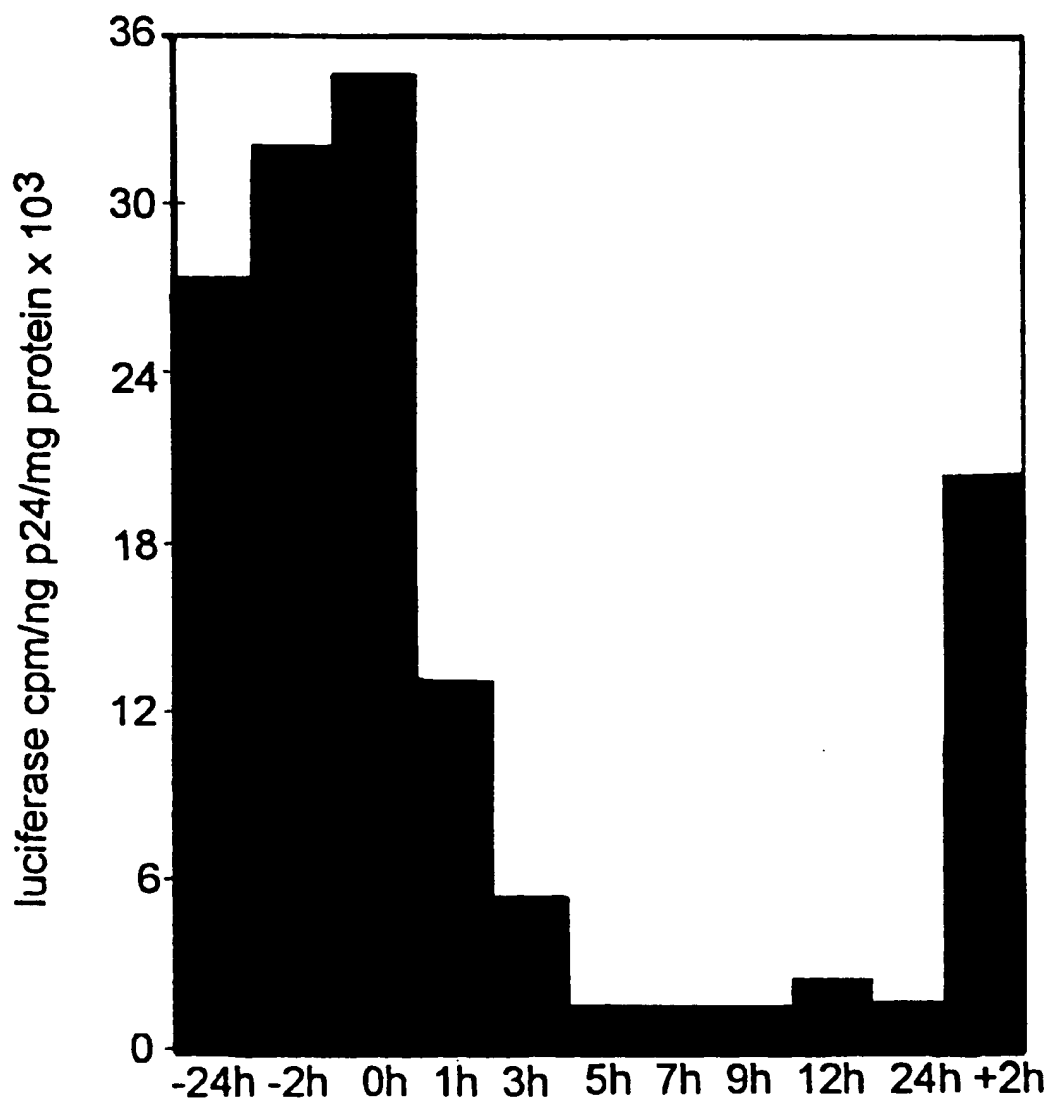


FIG. 3B

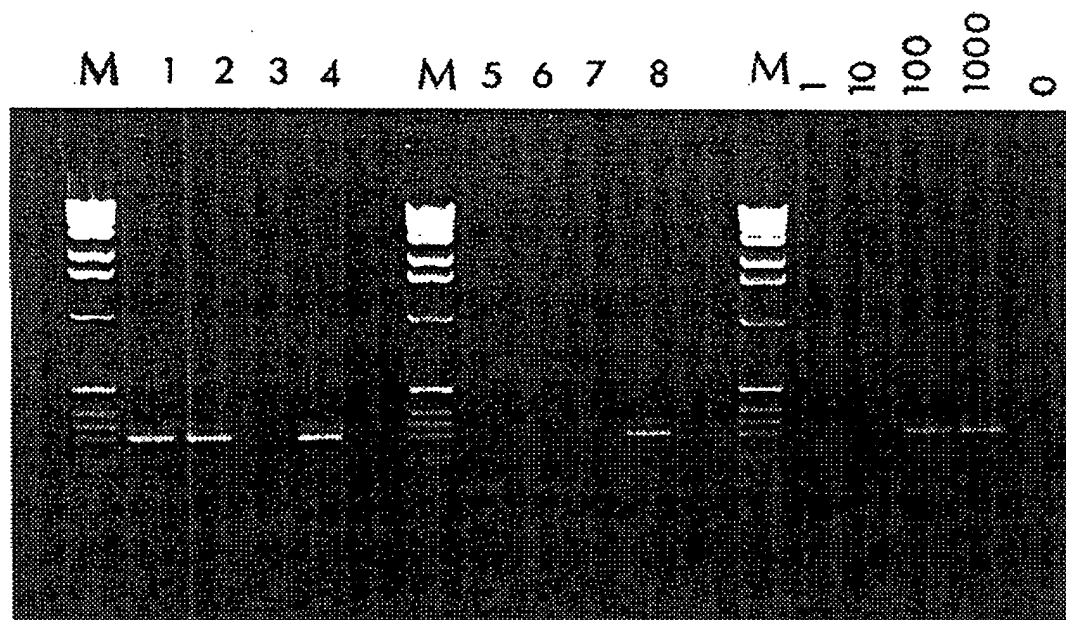


FIG. 4

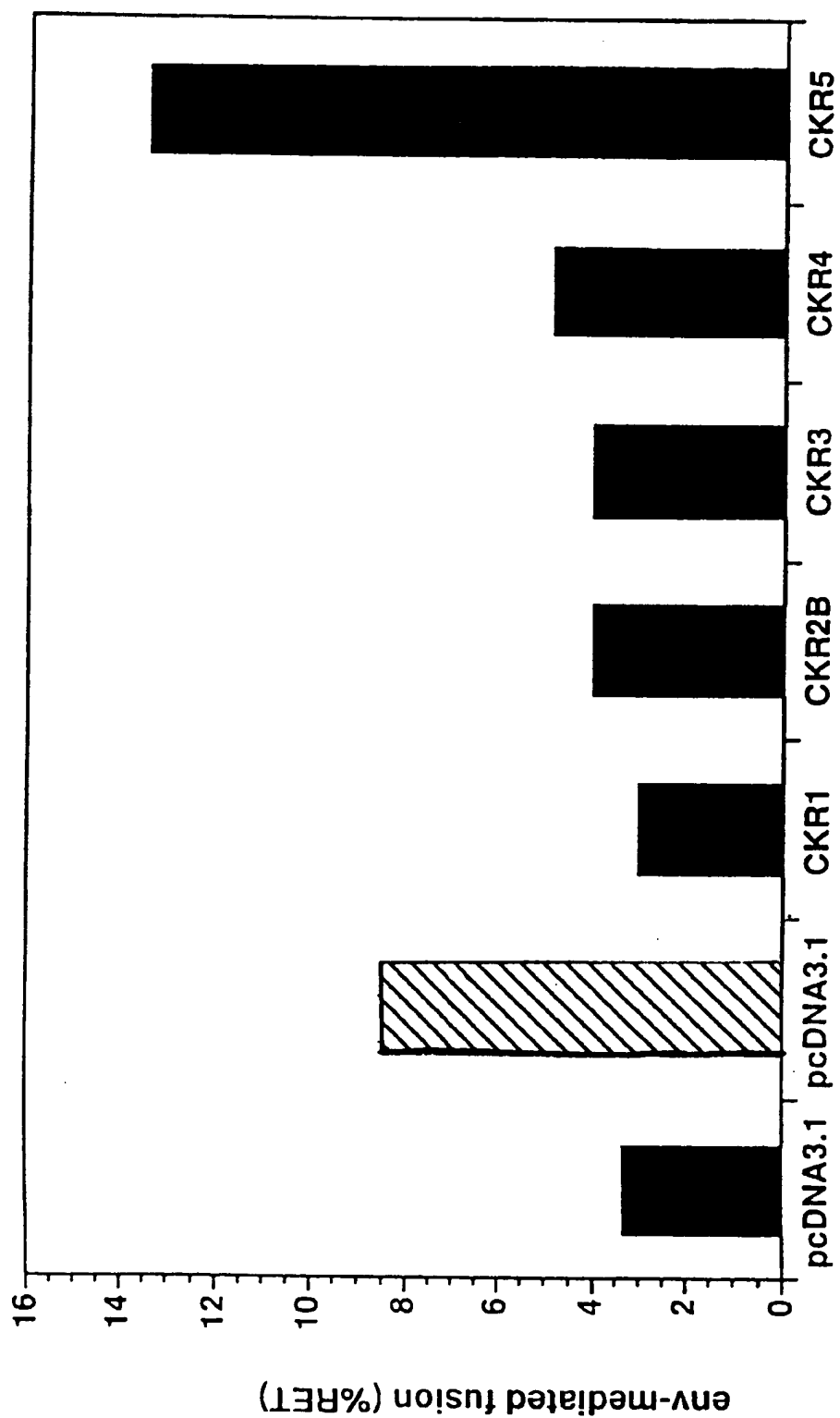
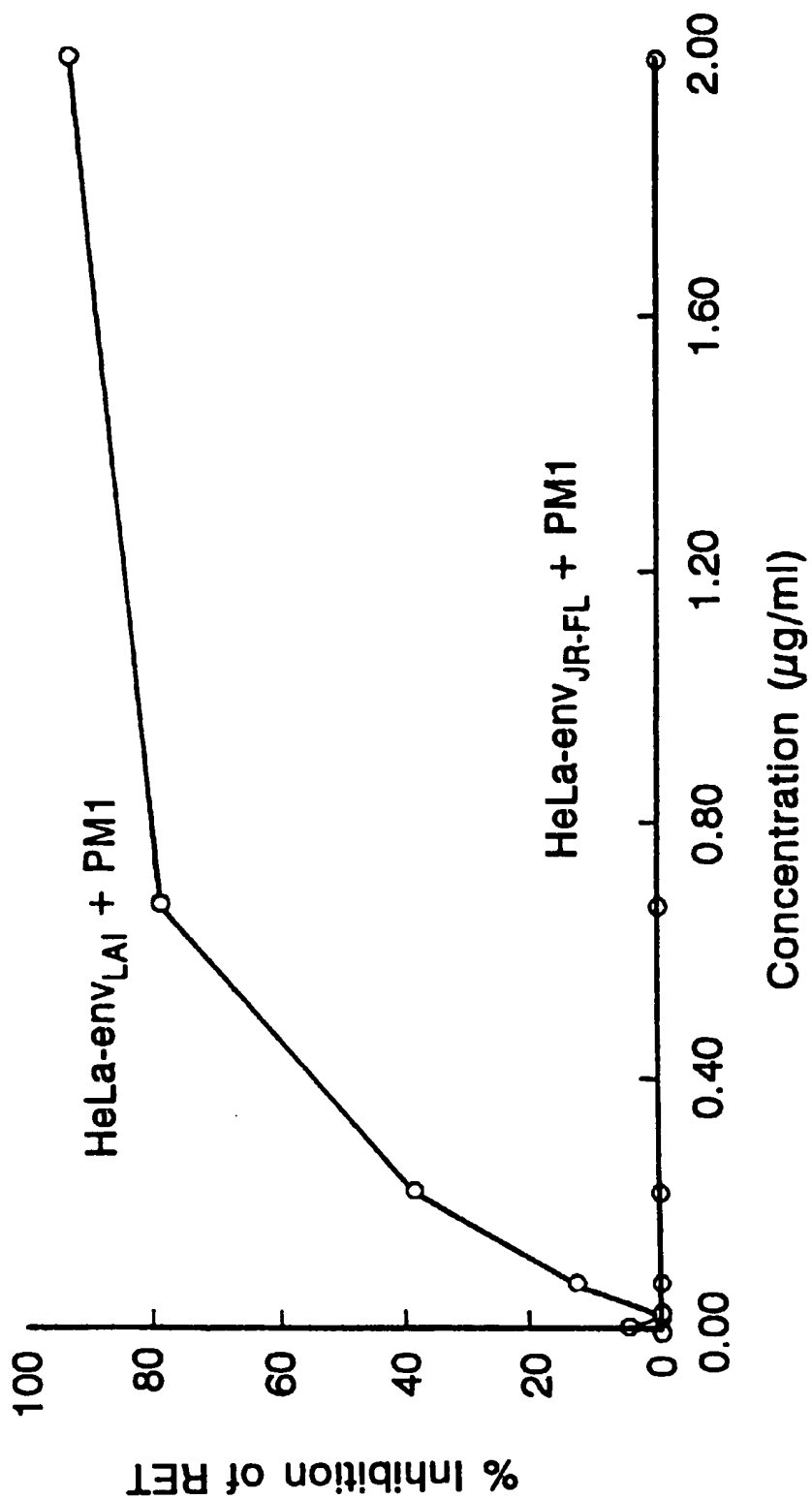


FIG. 5



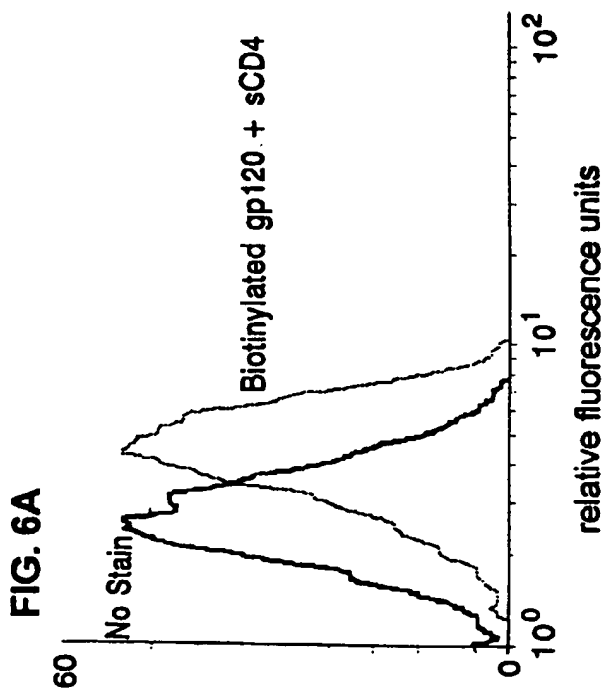
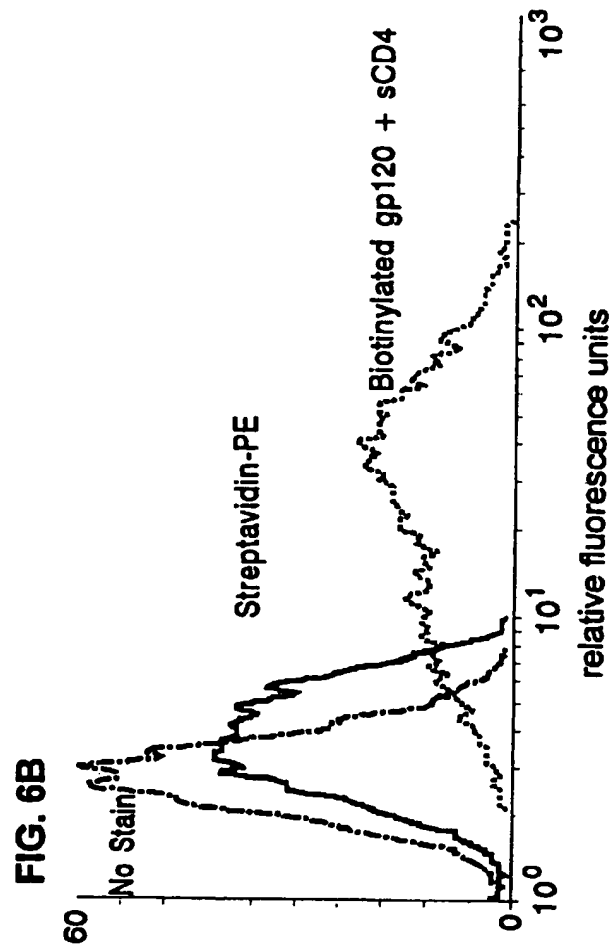
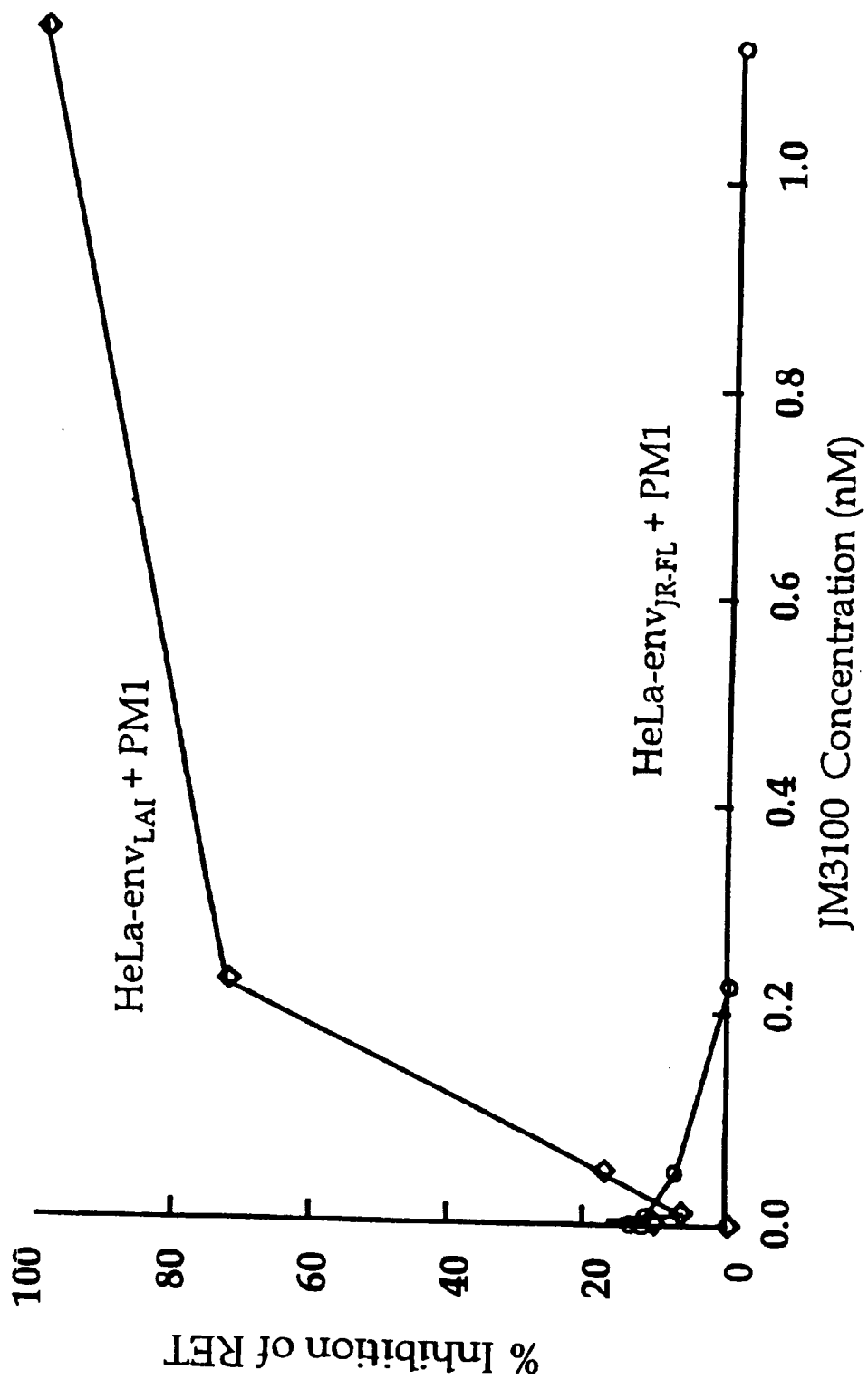


FIG. 7



## METHOD FOR PREVENTING HIV-1 INFECTION OF CD4<sup>+</sup> CELLS

This application is a continuation-in-part of U.S. Ser. No. 08/831,823, filed Apr. 2, 1997, which claims priority of U.S. Provisional Application No. 60/019,715, filed Jun. 14, 1996, now abandoned, and U.S. Provisional Application No. 60/014,532, filed Apr. 2, 1996, now abandoned. The contents of the applications being referred to above are incorporated by reference into this application.

Throughout this application, various references are referred to within parentheses. Disclosures of these publications in their entireties are hereby incorporated by reference into this application to more fully describe the state of the art to which this invention pertains. Full bibliographic citation for these references may be found at the end of each series of experiments.

### BACKGROUND OF THE INVENTION

Chemokines are a family of related soluble proteins of molecular weight between 8 and 10 KDa, secreted by lymphocytes and other cells, which bind receptors on target cell surfaces resulting in the activation and mobilization of leukocytes, for example in the inflammatory process. Recently, Cocchi et al. demonstrated that the chemokines RANTES, MIP-1 $\alpha$  and MIP-1 $\beta$  are factors produced by CD8<sup>+</sup> T lymphocytes which inhibit infection by macrophage-tropic primary isolates of HIV-1, but not infection by laboratory-adapted strains of the virus (1). These chemokines are members of the C—C group of chemokines, so named because they have adjacent cysteine residues, unlike the C-X-C group which has a single amino acid separating these residues (2). While Cocchi et al. found that expression of HIV-1 RNA was suppressed by treatment with the chemokines, they did not identify the site of action of these molecules.

A resonance energy transfer (RET) assay of HIV-1 envelope glycoprotein-mediated membrane fusion was used to determine whether fusion mediated by the envelope glycoprotein from the primary macrophage-tropic isolate of HIV-1<sub>JR-FL</sub> would be specifically inhibited by chemokines, when compared with fusion mediated by the envelope glycoprotein from the laboratory-adapted T lymphotropic strain HIV-1<sub>LAI</sub>. As described below, it was demonstrated that this is indeed the case. This demonstrates that some chemokine receptors are fusion accessory molecules required for HIV-1 infection. Previous studies have indicated that unidentified cell surface molecules are required for virus entry in addition to the HIV-1 receptor, CD4. While CD4 is required for HIV-1 attachment, the accessory molecules are required for the membrane fusion step of entry. These accessory molecules are generally expressed only on human cells, so HIV-1 does not infect non-human CD4<sup>+</sup> cells (3–6). Moreover it is possible to complement non-human CD4<sup>+</sup> cells by fusing them (using polyethylene glycol) with CD4<sup>+</sup> human cells, resulting in a heterokaryon which is a competent target for HIV-1 envelope-mediated membrane fusion (7,8). These studies have been performed using laboratory-adapted T lymphotropic strains of the virus.

In some cases, it appears that fusion accessory molecules are found on a subset of human CD4<sup>+</sup> cells and are required for infection by HIV-1 isolates with particular tropisms. For example, macrophage-tropic primary strains of HIV-1 such as HIV-1<sub>JR-FL</sub> may have different requirements for accessory molecules compared with laboratory-adapted T lymphotropic strains such as HIV-1<sub>LAI</sub>. This phenomenon may explain differences in tropism between HIV-1 strains.

The current invention comprises a series of new therapeutics for HIV-1 infection. It was demonstrated for the first time that chemokines act at the fusion step of HIV-1 entry and specifically inhibit membrane fusion mediated by the envelope glycoprotein of primary macrophage-tropic primary viral isolates, not laboratory-adapted T lymphotropic strains of the virus. Primary macrophage-tropic isolates of the virus are of particular importance since they are the strains usually involved in virus transmission, and may have particular importance in the pathogenesis of HIV-1 infection.

These results were obtained using a resonance energy transfer (RET) assay of HIV-1 envelope-mediated membrane fusion. Moreover, this assay is used to identify non-chemokines, including fragments of chemokines and modified chemokines, that inhibit HIV-1 envelope glycoprotein-mediated membrane fusion and thereby neutralize the virus, yet do not induce an inflammatory response.

### SUMMARY OF THE INVENTION

This invention provides a method for inhibiting fusion of HIV-1 to CD4<sup>+</sup> cells which comprises contacting CD4<sup>+</sup> cells with a non-chemokine agent capable of binding to a chemokine receptor in an amount and under conditions such that fusion of HIV-1 to the CD4<sup>+</sup> cells is inhibited.

This invention also provides a method for inhibiting HIV-1 infection of CD4<sup>+</sup> cells which comprises contacting CD4<sup>+</sup> cells with a non-chemokine agent capable of binding to a chemokine receptor in an amount and under conditions such that fusion of HIV-1 to the CD4<sup>+</sup> cells is inhibited, thereby inhibiting the HIV-1 infection.

This invention further provides non-chemokine agents capable of binding to the chemokine receptor and inhibiting fusion of HIV-1 to CD4<sup>+</sup> cells.

This invention provides an agent which is capable of binding to fusin and inhibiting infection. In an embodiment, the agent is an oligopeptide. In another embodiment, the agent is a polypeptide. In still another embodiment, the agent is an antibody or a portion of an antibody. In a separate embodiment, the agent is a nonpeptidyl agent.

In addition, this invention provides pharmaceutical compositions comprising an amount of the above non-chemokine agents or agents capable of binding to fusin effective to inhibit fusion of HIV-1 to CD4<sup>+</sup> cells and a pharmaceutically acceptable carrier.

This invention provides a composition of matter capable of binding to the chemokine receptor and inhibiting fusion of HIV-1 to CD4<sup>+</sup> cells comprising a non-chemokine agent linked to a ligand capable of binding to a cell surface receptor of the CD4<sup>+</sup> cells other than the chemokine receptor such that the binding of the non-chemokine agent to the chemokine receptor does not prevent the binding of the ligand to the other receptor.

This invention also provides a pharmaceutical composition comprising an amount of the above-described composition of matter effective to inhibit fusion of HIV-1 to CD4<sup>+</sup> cells and a pharmaceutically acceptable carrier.

This invention provides a composition of matter capable of binding to the chemokine receptor and inhibiting fusion of HIV-1 to CD4<sup>+</sup> cells comprising a non-chemokine agent linked to a compound capable of increasing the in vivo half-life of the non-chemokine agent.

This invention also provides a pharmaceutical composition comprising an amount of a composition of matter comprising a non-chemokine agent linked to a compound



capable of increasing the in vivo half-life of the non-chemokine agent effective to inhibit fusion of HIV-1 to CD4<sup>+</sup> cells and a pharmaceutically acceptable carrier.

This invention provide methods for reducing the likelihood of HIV-1 infection in a subject comprising administering an above-described pharmaceutical composition to the subject. This invention also provides methods for treating HIV-1 infection in a subject comprising administering an above-described pharmaceutical composition to the subject.

This invention also provides methods for determining whether a non-chemokine agent is capable of inhibiting the fusion of HIV-1 to a CD4<sup>+</sup> cell which comprise: (a) contacting (i) a CD4<sup>+</sup> cell which is labeled with a first dye and (ii) a cell expressing the HIV-1 envelope glycoprotein on its surface which is labeled with a second dye, in the presence of an excess of the agent under conditions permitting the fusion of the CD4<sup>+</sup> cell to the cell expressing the HIV-1 envelope glycoprotein on its surface in the absence of the agent, the first and second dyes being selected so as to allow resonance energy transfer between the dyes; (b) exposing the product of step (a) to conditions which would result in resonance energy transfer if fusion has occurred; and (c) determining whether there is a reduction of resonance energy transfer, when compared with the resonance energy transfer in the absence of the agent, a decrease in transfer indicating that the agent is capable of inhibiting fusion of HIV-1 to CD4<sup>+</sup> cells.

#### BRIEF DESCRIPTION OF THE FIGURES

FIGS. 1A–C. Membrane fusion mediated by the HIV-1<sub>JR-FL</sub> envelope glycoprotein is inhibited by RANTES, MIP-1 $\alpha$  and MIP-1 $\beta$ .

% RET resulting from the fusion of PM1 cells and HeLa-env<sub>JR-FL</sub> (■) or HeLa-env<sub>LAI</sub> (◆) was measured in the presence and absence of recombinant human chemokines at a range of concentrations: RANTES (80–2.5 ng/ml), MIP-1 $\alpha$  (400–12.5 ng/ml) and MIP-1 $\beta$  (200–6.25 ng/ml), as indicated. Chemokines were added simultaneously with the cells at the initiation of a four hour incubation. Data are representative of more than three independent experiments which were run in duplicate. The percent inhibition of RET is defined as follows:

$$\% \text{ Inhibition} = 100 - \frac{(\text{Max RET} - \text{Min RET}) - (\text{Exp RET} - \text{Min RET})}{(\text{Max RET} - \text{Min RET})}$$

where Max RET is the % RET value obtained at four hours with HeLa-env cells and CD4-expressing cells in the absence of an inhibitory compound; Exp RET is the FRET value obtained for the same cell combination in the presence of an inhibitory compound and Min RET is the background % RET value obtained using HeLa cells in place of HeLa envelope-expressing cells.

FIG. 2A–B. CD4:HIV-1 gp120 binding in the presence of human chemokines.

The binding of soluble human CD4 to HIV-1<sub>LAI</sub> and HIV-1<sub>JR-FL</sub> gp120 was determined in an ELISA assay in the presence and absence of the monoclonal antibody OKT4A or recombinant human chemokines at a range of concentrations, identical to those used in the RET inhibition studies of FIG. 1: OKT4A (62–0.3 nM), RANTES (10.3–0.3 nM), MIP-1 $\alpha$  (53.3–2.9 nM), and MIP-1 $\beta$  (25.6–0.8 nM). Inhibitors were added simultaneously with biotinylated HIV-1 gp120 to soluble CD4 coated microtiter plates (Dynatech Laboratories, Inc., Chantilly, Va.). Following a two hour incubation at room temperature and extensive washing, an incubation with streptavidin-horseradish peroxidase was performed for one hour at room temperature.

Following additional washes, substrate was added and the OD at 492 nm determined in an ELISA plate reader. Data are representative of two independent experiments which were run in quadruplicate.

FIG. 3A–B. Specificity, time course and stage of  $\beta$ -chemokine inhibition of HIV-1 replication.

(a) PM1 cells ( $1 \times 10^6$ ) were preincubated with RANTES+ MIP-1 $\alpha$ +MIP-1 $\beta$  (R/M $\alpha$ /M $\beta$ ; 100 ng/ml of each) for 24 h (–24 h) or 2 h (–2 h), then washed twice with phosphate buffered saline (PBS). HIV-1 (BaL env-complemented) virus (50 ng of p24; see legend to Table 1) was added for 2 h, then the cells were washed and incubated for 48 h before measurement of luciferase activity in cell lysates as described previously (10,11). Alternatively, virus and R/M $\alpha$ /M $\beta$  were added simultaneously to cells, and at the indicated time points (1 h, 3 h, etc) the cells were washed twice in PBS, resuspended in culture medium and incubated for 48 h prior to luciferase assay. Time 0 represents the positive control, to which no  $\beta$ -chemokines were added. +2 h represents the mixture of virus with cells for 2 h prior to washing twice in PBS, addition of R/M $\alpha$ /M $\beta$  and continuation of the culture for a further 48 h before luciferase assay.

(b) PM1 cells ( $1 \times 10^6$ ) were infected with HIV-1 (500 pg p24) grown in CEM cells (NL4/3; lanes 1–4) or macrophages (ADA; lanes 5–8), in the presence of 500 ng/ml of RANTES (lanes 1 and 5) or MIP-1 $\beta$  (lanes 2 and 6), or with no  $\beta$ -chemokine (lanes 4 and 8). Lanes 3 and 7 are negative controls (no virus). All viral stocks used for the PCR assay were treated with DNase for 30 min at 37° C., and tested for DNA contamination before use. After 2 h, the cells were washed and resuspended in medium containing the same  $\beta$ -chemokines for a further 8 h. DNA was then extracted from infected cells using a DNA/RNA isolation kit (US Biochemicals). First round nested PCR was performed with primers:

U3+,  
5'-CAAGGCTACTTCCCTGATTGGCAGAACTACACACCAGG-3' (SEQ ID NO:1) preGag,  
5'-AGCAAGCCGAGTCCTGCGTCGAGAG-3' (SEQ ID NO:2) and the second round with primers: LTR-test,  
5'-GGGACTTCCGCTGGGGACTTTC 3' (SEQ ID NO:3) LRC2,  
5'-CCTGTTTCGGGCGCCACTGCTAGAGATTTCAC 3' (SEQ ID NO:4) in a Perkin Elmer 2400 cycler with the following amplification cycles: 94° C. for 5 min, 35 cycles of 94° C. for 30s, 55° C. for 30s, 72° C. for 30s, 72° C. for 7 min. M indicates 1 kb DNA ladder; 1, 10, 100, 1000 indicate number of reference plasmid (pAD8) copies. The assay can detect 100 copies of reverse transcripts.

FIG. 4: HIV-1 env-mediated membrane fusion of cells transiently expressing C–C CKR-5.

Membrane fusion mediated by  $\beta$ -chemokine receptors expressed in HeLa cells was demonstrated as follows: Cells were transfected with control plasmid pcDNA3.1 or plasmid pcDNA3.1-CKR constructs using lipofectin (Gibco BRL). The pcDNA3.1 plasmid carries a T7-polymerase promoter and transient expression of  $\beta$ -chemokine receptors was boosted by infecting cells with  $1 \times 10^7$  pfu of vaccinia encoding the T7-polymerase (vFT7.3) 4 h post-lipofection (9). Cells were then cultured overnight in R18-containing media and were tested for their ability to fuse with HeLa-JR-FL cells (filled columns) or HeLa-BRU cells (hatched column) in the RET assay. The FRET with control HeLa cells was between 3% and 4% irrespective of the transfected plasmid.

FIG. 5 Membrane fusion mediated by the HIV<sub>LAI</sub> envelope glycoprotein is inhibited by SDF-1.

% RET resulting from the fusion of PM1 cells and HeLa-env<sub>JR-FL</sub> or HeLa-env<sub>LAI</sub> cells (as indicated on the

graph) was measured in the presence of recombinant SDF-1 $\alpha$  (Gryphon Science, San Francisco) at the indicated concentrations. Experimental method as described in the legend to FIG. 1.

FIGS. 6A–B. Flow cytometric analysis of the binding of sCD4-gp120 complexes to (a)CCR5<sup>+</sup> and (b)CCR5<sup>+</sup> L1.2 cells, a murine pre-B lymphoma line. Cells are incubated for 15 min. with equimolar (~100 nM) mixtures of sCD4 and biotinylated HIV-1<sub>JR-FL</sub> gp120 and then stained with a streptavidin-phycoerythrin conjugate, fixed with 2% paraformaldehyde, and analyzed by FACS. Cell number is plotted on the y-axis.

FIG. 7. Inhibition of HIV-1 envelope-mediated cell fusion by the bicyclam JM3100, measured using the RET assay, with the cell combinations indicated.

#### DETAILED DESCRIPTION OF THE INVENTION

This invention provides a method for inhibiting fusion of HIV-1 to CD4<sup>+</sup> cells which comprises contacting CD4<sup>+</sup> cells with a non-chemokine agent capable of binding to a chemokine receptor in an amount and under conditions such that fusion of HIV-1 to the CD4<sup>+</sup> cells is inhibited.

This invention also provides a method for inhibiting HIV-1 infection of CD4<sup>+</sup> cells which comprises contacting CD4<sup>+</sup> cells with a non-chemokine agent capable of binding to a chemokine receptor in an amount and under conditions such that fusion of HIV-1 to the CD4<sup>+</sup> cells is inhibited, thereby inhibiting the HIV-1 infection.

In this invention, a chemokine means RANTES, MIP-1- $\alpha$ , MIP-1- $\beta$  or another chemokine which blocks HIV-1 infection. A chemokine receptor means a receptor capable of binding RANTES, MIP-1- $\alpha$ , MIP-1- $\beta$  or another chemokine which blocks HIV-1 infection. Such chemokine receptor includes but not limited to CCR5, CXCR4, CCR3 and CCR-2b.

Throughout this application, the receptor "fusin" is also named CXCR4 and the chemokine receptor C—C CKR5 is also named CCR5.

The HIV-1 used in this application unless specified will mean clinical or primary or field isolates or HIV-1 viruses which maintain their clinical characteristics. The HIV-1 clinical isolates may be passaged in primary peripheral blood mononuclear cells. The HIV-1 clinical isolates may be macrophage-trophic.

The non-chemokine agents of this invention are capable of binding to chemokine receptors and inhibiting fusion of HIV-1 to CD4<sup>+</sup> cells. The non-chemokine agents include, but are not limited to, chemokine fragments and chemokine derivatives and analogues, but do not include naturally occurring chemokines. The non-chemokine agents include multimeric forms of the chemokine fragments and chemokine derivatives and analogues or fusion molecules which contain chemokine fragments, derivatives and analogues linked to other molecules.

The non-chemokine agents do not include bicyclams and their derivatives as described in U.S. Pat. No. 5,021,409, issued Jun. 4, 1991, the content of which is incorporated by reference into this application. Some bicyclam derivatives have been previously described with antiviral activities (15, 16).

In an embodiment of this invention, the non-chemokine agent is an oligopeptide. In another embodiment, the non-chemokine agent is a polypeptide. In still another embodiment, the non-chemokine agent is an antibody or a portion thereof. Antibodies against the chemokine receptor

may easily be generated by routine experiments. It is also within the level of ordinary skill to synthesize fragments of the antibody capable of binding to the chemokine receptor. In a further embodiment, the non-chemokine agent is a nonpeptidyl agent.

Non-chemokine agents which are purely peptidyl in composition can be either chemically synthesized by solid-phase methods (Merrifield, 1966) or produced using recombinant technology in either prokaryotic or eukaryotic systems. The synthetic and recombinant methods are well known in the art.

Non-chemokine agents which contain biotin or other nonpeptidyl groups can be prepared by chemical modification of synthetic or recombinant chemokines or non-chemokine agents. One chemical modification method involves periodate oxidation of the 2-amino alcohol present on chemokines or non-chemokine agents possessing serine or threonine as their N-terminal amino acid (Geophegan and Stroh, 1992). The resulting aldehyde group can be used to link peptidyl or non-peptidyl groups to the oxidized chemokine or non-chemokine agent by reductive amination, hydrazine, or other chemistries well known to those skilled in the art.

As used herein, a N-terminus of a protein should mean the terminus of the protein after it has been processed. In case of a secretory protein which contains a cleavable signal sequence, the N-terminus of a secretory protein should be the terminus after the cleavage of a signal peptide.

This invention provides a method of identifying these non-chemokine agents. One way of identifying such agents, including non-peptidyl agents, that bind to a chemokine receptor and inhibit fusion of HIV-1 to CD4<sup>+</sup> cells is to use the following assay: 1) Incubate soluble CD4 with biotinylated gp120 from HIV-1<sub>JR-FL</sub> or HIV-1<sub>LAJ</sub>; 2) Incubate this complex with CCR5 or CXCR4-expressing cells (for HIV-1<sub>JR-FL</sub> or HIV-1<sub>LAJ</sub> gp120s, respectively) that do not express CD4, in the presence or absence of a candidate inhibitor; 3) Wash and then incubate with streptavidin-phycoerythrin; and 4) Wash and then measure the amount of bound gp120 using a flow cytometer or fluorometer and calculate the degree of inhibition of binding by the inhibitor.

Alternative methods to detect bound gp120 can also be used in place of the biotinylated gp120-streptavidin-phycoerythrin method described above. For example, peroxidase-conjugated gp120 could be used in place of the biotinylated gp120 and binding detected using an appropriate colorimetric substrate for peroxidase, with a spectrometric readout.

This invention further provides the non-chemokine agents identified by the above methods.

This invention provides a non-chemokine agent capable of binding to the chemokine receptor and inhibiting fusion of HIV-1 to CD4<sup>+</sup> cells with the proviso that the agent is not a known bicyclam or its known derivatives. In an embodiment, the non-chemokine is a polypeptide. In a further embodiment, this polypeptide is a fragment of the chemokine RANTES (Gong et al., 1996). In a still further embodiment, the polypeptide may also comprise the RANTES sequence with deletion of the N-terminal amino acids of said sequence. The deletion may be the first eight N-terminal amino acids of the RANTES sequence (SEQ ID NO:5).

In a separate embodiment, the polypeptide may comprise the MIP-1 $\beta$  sequence with deletion of the N-terminal amino acids of said sequence. The deletion may be the first seven, eight, nine or ten N-terminal amino acids of the MIP-1 $\beta$  sequence.

In another embodiment of non-chemokine agent, the polypeptide comprises the MIP-1 $\beta$  sequence with the N-terminal sequence modified by addition of an amino acid or oligopeptide. In a separate embodiment, the polypeptide comprises the MIP-1 $\beta$  sequence with the N-terminal sequence modified by removing the N-terminal alanine and replaced it by serine or threonine and additional amino acid or oligopeptide or nonpeptidyl moiety. In a further embodiment, the additional amino acid is methionine.

As described *infra* in the section of Experimental Details, a cofactor for HIV-1 fusion and entry was identified and designated "fusin" (Feng et al., 1996). This invention provides an agent which is capable of binding to fusin and inhibiting infection. In an embodiment, the agent is an oligopeptide. In another embodiment, the agent is an polypeptide.

In a further embodiment, the polypeptide comprises SDF-1 with deletion of the N-terminal amino acids of said sequence. The deletion may be the first six, seven, eight, or nine N-terminal amino acids of the SDF-1 sequence.

This invention also provides the above non-chemokine agent, wherein the polypeptide comprises SDF-1 sequence with the N-terminal sequence modified to produce antagonistic effect to SDF-1. One modification is to replace the N-terminal glycine of SDF-1 by serine and derivatized with biotin. Another modification is to replace the N-terminal glycine of SDF-1 by serine and derivatized with methionine. A further modification is to add the N-terminus of SDF-1 with a methionine before the terminal glycine.

In still another embodiment, the agent is an antibody or a portion of an antibody. In a separate embodiment, the agent is a nonpeptidyl agent.

The agents capable of binding to fusin may be identified by screening different compounds for their capability to bind to fusin *in vitro*.

A suitable method has been described by Fowlkes, et al. (1994), international application number: PCT/US94/03143, international publication number: WO 94/23025, the content of which is incorporated by reference into this application. Briefly, yeast cells having a pheromone system are engineered to express a heterologous surrogate of a yeast pheromone system protein. The surrogate incorporates fusin and under some conditions performs in the pheromone system of the yeast cell a function naturally performed by the corresponding yeast pheromone system protein. Such yeast cells are also engineered to express a library of peptides whereby a yeast cell containing a peptide which binds fusin exhibits modulation of the interaction of surrogate yeast pheromone system protein with the yeast pheromone system and this modulation is a selectable or screenable event. Similar approaches may be used to identify agents capable of binding to both fusin and the chemokine receptor C—C CKR-5.

This invention also provides pharmaceutical compositions comprising an amount of such non-chemokine agents or agents capable of binding to fusin effective to inhibit fusion of HIV-1 to CD4<sup>+</sup> cells and a pharmaceutically acceptable carrier.

Pharmaceutically acceptable carriers are well known to those skilled in the art. Such pharmaceutically acceptable carriers may be aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, saline and

buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers such as those based on Ringer's dextrose, and the like. Preservatives and other additives may also be present, such as, for example, antimicrobials, antioxidants, chelating agents, inert gases and the like.

This invention provides a composition of matter capable of binding to the chemokine receptor and inhibiting fusion of HIV-1 to CD4<sup>+</sup> cells comprising a non-chemokine agent linked to a ligand capable of binding to a cell surface receptor of the CD4<sup>+</sup> cells other than the chemokine receptor such that the binding of the non-chemokine agent to the chemokine receptor does not prevent the binding of the ligand to the other receptor. In an embodiment, the cell surface receptor is CD4. In another embodiment, the ligand is an antibody or a portion of an antibody.

This invention also provides a pharmaceutical composition comprising an amount of an above-described composition of matter effective to inhibit fusion of HIV-1 to CD4<sup>+</sup> cells and a pharmaceutically acceptable carrier.

This invention provides a composition of matter capable of binding to the chemokine receptor and inhibiting fusion of HIV-1 to CD4<sup>+</sup> cells comprising a non-chemokine agent linked to a compound capable of increasing the *in vivo* half-life of the non-chemokine agent. In an embodiment, the compound is polyethylene glycol.

This invention also provides a pharmaceutical composition comprising an amount of a composition of matter comprising a non-chemokine agent linked to a compound capable of increasing the *in vivo* half-life of the non-chemokine agent effective to inhibit fusion of HIV-1 to CD4<sup>+</sup> cells and a pharmaceutically acceptable carrier.

This invention provide methods for reducing likelihood of HIV-1 infection in a subject comprising administering the above-described pharmaceutical compositions to the subject. This invention also provides methods for treating HIV-1 infection in a subject comprising administering the above-described pharmaceutical compositions to the subject.

This invention also provides methods for determining whether a non-chemokine agent is capable of inhibiting the fusion of HIV-1 to a CD4<sup>+</sup> cell which comprise: (a) contacting (i) a CD4<sup>+</sup> cell which is labeled with a first dye and (ii) a cell expressing the HIV-1 envelope glycoprotein on its surface which is labeled with a second dye, in the presence of an excess of the agent under conditions permitting the fusion of the CD4<sup>+</sup> cell to the cell expressing the HIV-1 envelope glycoprotein on its surface in the absence of the agent, the first and second dyes being selected so as to allow resonance energy transfer between the dyes; (b) exposing the product of step (a) to conditions which would result in resonance energy transfer if fusion has occurred; and (c) determining whether there is a reduction of resonance energy transfer, when compared with the resonance energy transfer in the absence of the agent, a decrease in transfer indicating that the agent is capable of inhibiting fusion of HIV-1 to CD4<sup>+</sup> cells.

HIV-1 only fuses with appropriate CD4<sup>+</sup> cells. For example, laboratory-adapted T lymphotropic HIV-1 strains fuse with most CD4<sup>+</sup> human cells. Clinical HIV-1 isolates do not fuse with most transformed CD4<sup>+</sup> human cell lines but do fuse with human primary CD4<sup>+</sup> cells such as CD4<sup>+</sup> T lymphocytes and macrophages. Routine experiments may be easily performed to determine whether the CD4<sup>+</sup> cell is appropriate for the above fusion assay.

As described in this invention, HIV-1 membrane fusion is monitored by a resonance energy transfer assay. The assay was described in the International Application Number, PCT/US94/14561, filed Dec. 16, 1994 with International Publication Number WO 95/16789. This assay is further elaborated in a United States co-pending application no. 08/475,515, filed Jun. 7, 1995. The contents of these applications are hereby incorporated by reference into this application.

In an embodiment of the above method, the non-chemokine agent is an oligopeptide. In another embodiment, the non-chemokine agent is a polypeptide. In still another embodiment, the agent is an antibody or a portion thereof. In a further embodiment, the non-chemokine agent is a non-peptidyl agent.

In a separate embodiment, the CD4<sup>+</sup> cell is a PM1 cell. In another embodiment, the cell expressing the HIV-1 envelope glycoprotein is a HeLa cell expressing HIV-1<sub>JR-FL</sub> gp120/gp41.

This invention provides a method for determining whether an agent is capable of inhibiting HIV-1 infection comprising steps of: (a) fixing a chemokine receptor on a solid matrix; (b) contacting the agent with the fixed chemokine receptor under conditions permitting the binding of the agent to the chemokine receptor; (c) removing the unbound agent; (d) contacting the fixed chemokine receptor resulting in step (c) with a gp120 in the presence of CD4 under conditions permitting the binding of the gp120/CD4 complex and the chemokine receptor in the absence of the agent; (e) measuring the amount of bound gp120/CD4 complex; and (f) comparing the amount determined in step (d) with the amount determined in the absence of the agent, a decrease of the amount indicating that the agent is capable of inhibiting HIV-1 infection.

This invention also provides a method for determining whether an agent is capable of inhibiting HIV-1 infection comprising steps of: (a) fixing a chemokine receptor on a solid matrix; (b) contacting the agent with the fixed chemokine receptor; (c) contacting the mixture in step (b) with a gp120 in the presence of CD4 under conditions permitting the binding of the gp120/CD4 complex and the chemokine receptor in the absence of the agent; (d) measuring the amount of bound gp120/CD4 complex; and (e) comparing the amount determined in step (d) with the amount determined in the absence of the agent, a decrease of the amount indicating that the agent is capable of inhibiting HIV-1 infection.

This invention also provides a method for determining whether an agent is capable of inhibiting HIV-1 infection comprising steps of: (a) fixing a gp120/CD4 complex on a solid matrix; (b) contacting the agent with the fixed gp120/CD4 complex under conditions permitting the binding of the agent to the gp120/CD4 complex; (c) removing unbound agent; (d) contacting the fixed gp120/CD4 complex resulting from step (c) with a chemokine receptor under conditions permitting the binding of the gp120/CD4 complex and the chemokine receptor in the absence of the agent; (e) measuring the amount of bound chemokine receptor; and (f) comparing the amount determined in step (e) with the amount determined in the absence of the agent, a decrease of the amount indicating that the agent is capable of inhibiting HIV-1 infection.

This invention provides a method for determining whether an agent is capable of inhibiting HIV-1 infection comprising steps of: (a) fixing a gp120/CD4 on a solid matrix; (b) contacting the agent with the fixed gp120/CD4

complex; (c) contacting the mixture in step (b) with a chemokine receptor under conditions permitting the binding of the gp120/CD4 complex and the chemokine receptor in the absence of the agent; (d) measuring the amount of bound chemokine receptor; (e) comparing the amount determined in step (d) with the amount determined in the absence of the agent, a decrease of the amount indicating that the agent is capable of inhibiting HIV-1 infection.

As used in these assays, CD4 include soluble CD4, fragments of CD4 or polypeptides incorporating the gp120 binding site of CD4 capable of binding gp120 and enabling the binding of gp120 to the appropriate chemokine receptor.

As used in these assay gp120 is the gp120 from an appropriate strain of HIV-1. For example, gp120 from the macrophage tropic clinical isolate HIV-1<sub>JR-FL</sub> will bind to the chemokine receptor CCR5, whereas gp120 from the laboratory adapted T-tropic strain HIV-1<sub>LAI</sub> will bind to the chemokine receptor CXCR4.

In a preferred embodiment of the above methods, the CD4 is a soluble CD4. The chemokine receptor which may be used in the above assay includes CCR5, CXCR4, CCR3 and CCR-2b.

In an embodiment, the chemokine receptor is expressed on a cell. In a preferred embodiment, the cell is a L1.2 cell. In a separate embodiment, the chemokine receptor is purified and reconstituted in liposomes. Such chemokine receptor embedded in the lipid bilayer of liposomes retains the gp120 binding activity of the receptor.

The gp120, CD4 or both may be labelled with a detectable marker in the above assays. Markers including radioisotope or enzymes such as horse radish peroxidase may be used in this invention.

In an embodiment, the gp120 or CD4 or the chemokine receptor is labelled with biotin. In a further embodiment, the biotinylated gp120, or CD4 or the chemokine receptor is detected by: (i) incubating with streptavidin-phycoerythrin, (ii) washing the incubated mixture resulting from step (i), and (iii) measuring the amount of bound gp120 using a plate reader, exciting at 530 nm, reading emission at 590 nm.

This invention also provides an agent determined to be capable of inhibiting HIV-1 infection by the above methods, which is previously unknown.

This invention also provides a pharmaceutical composition comprising the agent determined to be capable of inhibiting HIV-1 infection by the above methods and a pharmaceutically acceptable carrier. In an embodiment, the agent is an oligopeptide. In another embodiment, the agent is a polypeptide. In a still another embodiment, the agent is a nonpeptidyl agent.

This invention also provides a molecule capable of binding to the chemokine receptor CCR5 and inhibiting fusion of HIV-1 to CD4<sup>+</sup> cells comprising the above determined agent linked to a compound capable of increasing the in vivo half-life of the non-chemokine agent. In an embodiment, the compound is polyethylene glycol. This invention also provides a pharmaceutical composition comprising an amount of the above molecule effective to inhibit HIV-1 infection and a pharmaceutically acceptable carrier.

This invention provides a method for reducing the likelihood of HIV-1 infection in a subject comprising administering the above pharmaceutical compositions to the subject.

This invention provides a method for treating HIV-1 infection in a subject comprising administering the above pharmaceutical composition to the subject.

This invention will be better understood by reference to the Experimental Details which follow, but those skilled in

the art will readily appreciate that the specific experiments detailed are only illustrative of the invention as described more fully in the claims which follow thereafter.

#### EXPERIMENTAL DETAILS

##### First Series of Experiments

- 1) Chemokines inhibit fusion mediated by the envelope glycoprotein from a macrophage-tropic primary isolate of HIV-1 but not from a laboratory-adapted T-lymphotrophic strain of the virus

The chemokines RANTES, MIP-1 $\alpha$  and MIP-1 $\beta$  were obtained from R & D systems (Minneapolis, Minn.). They were tested in the RET assay for ability to inhibit fusion between HeLa-env<sub>JR-FL</sub> cells (expressing gp120/gp41 from the macrophage tropic isolate HIV-1<sub>JR-FL</sub>) and PM1 cells, or for inhibition of fusion between HeLa-env<sub>LAI</sub> cells (expressing gp120/gp41 from the laboratory-adapted strain HIV-1<sub>LAI</sub>) and various CD4<sup>+</sup> T lymphocyte cell lines. As shown in FIG. 1, all three chemokines inhibited fusion mediated by the macrophage tropic virus envelope glycoprotein, but not that mediated by the laboratory-adapted strain envelope glycoprotein.

The ability of the chemokines to block the interaction between CD4 and HIV-1 gp120 which occurs at virus attachment was then tested. It was found that the chemokines did not inhibit this interaction (FIG. 2), demonstrating that their blockade of HIV-1 envelope glycoprotein-mediated membrane fusion occurs at the membrane fusion event itself, rather than the initial CD4-gp120 interaction which precedes fusion.

- 2) Non-chemokine peptides and derivatives that inhibit HIV-1 fusion

The non-chemokines include chemokine fragments and chemokine derivatives that are tested in the RET assay to determine which are active in inhibiting HIV-1 membrane fusion. Particular attention is focused on fragments or derivatives that inhibit HIV-1 fusion but do not activate leukocyte responses. These non-chemokines include:

- a) N-terminal derivatives of the chemokines. Addition of residues to the N-terminus of chemokines inhibits the function of these proteins without significantly reducing their ability to bind chemokine receptors. For example, Met-RANTES (RANTES with an N-terminal methionine) has been shown to be a powerful antagonist of native RANTES and is unable to induce chemotaxis or calcium mobilization in certain systems. The mechanism of antagonism appears to be competition for receptor binding (9). Similar results were found using other derivatives of the N terminus of RANTES (9) and also by N-terminal modification of other chemokines, such as IL-8 (a member of the C-X-C chemokines) (10). The current invention includes Met-RANTES and other chemokines derivatised by the addition of methionine, or other residues, to the N-terminus so that they inhibit fusion mediated by the envelope glycoprotein of HIV-1<sub>JR-FL</sub>, and inhibit infection by many isolates of HIV-1, yet do not activate the inflammatory response.
- b) Chemokines with N-terminal amino acids deleted: Chemokine antagonists have been generated by deleting amino acids in the N-terminal region. For example, deletion of up to 8 amino acids at the N-terminus of the chemokine MCP-1 (a member of the C-C chemokine group), ablated the bioactivity of the protein while allowing it to retain chemokine receptor binding and the ability to inhibit activity of native MCP-1 (11,12).

The current invention includes N-terminal deletants of RANTES, MIP-1 $\alpha$  and MIP-1 $\beta$ , lacking the biological activity of the native proteins, which inhibit HIV-1 fusion and HIV-1 infection.

- c) Other peptides: A series of overlapping peptides (e.g. of 20-67 residues) from all regions of RANTES, MIP-1 $\alpha$  and MIP-1 $\beta$  are screened by the same approaches to identify peptides which inhibit HIV-1 fusion most potently without activating leukocytes. Activation of leukocyte responses is measured following routine procedures (9, 10, 11, 12).

- 3) Cloning the chemokine receptors

Chemokine receptors required for HIV-1 fusion are cloned by the following strategy. First a cDNA library is made in a mammalian expression vector (e.g. pcDNA3.1 from Invitrogen Corp. San Diego, Calif.) using mRNA prepared from the PM1 cell line or CD4<sup>+</sup> T-lymphocytes or macrophages. Degenerate oligonucleotide probes are used to identify members of the cDNA library encoding members of the chemokine receptor family, for example following previously published methods (2). The vectors containing chemokine receptor cDNAs are then individually expressed in one of several mammalian cell lines which express human CD4 but do not fuse with HeLa-env<sub>JR-FL</sub> cells (e.g. HeLa-CD4, CHO-CD4 or COS-CD4) or HeLa-env<sub>LAI</sub> cells (e.g. CHO-CD4 or COS-CD4). Following analysis in the RET assay, clones which gain the ability to fuse with HeLa-env<sub>JR-FL</sub> or HeLa-env<sub>LAI</sub> are identified and the coding sequences recovered, for example by PCR amplification, following procedures well known to those skilled in the art. DNA sequencing is then performed to determine whether the cDNA recovered encodes a known chemokine receptor. Following expression of the receptor, monoclonal and polyclonal antibodies are prepared and tested for ability to inhibit infection by a panel of HIV-1 isolates.

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#### Second Series of Experiments

The replication of primary, non-syncytium-inducing (NSI) HIV-1 isolates in CD4<sup>+</sup> T-cells is inhibited by the C—C  $\beta$ -chemokines MIP-1 $\alpha$ , MIP-1 $\beta$  and RANTES (1,2), but T-cell line-adapted (TCLA) or syncytium-inducing (SI) primary strains are insensitive (2,3). The  $\beta$ -chemokines are small (8 kDa), related proteins active on cells of the lymphoid and monocyte lineage (4-8). Their receptors are members of the 7-membrane-spanning, G-protein-linked superfamily, one of which (the LESTR orphan receptor) has been identified as the second receptor for TCLA HIV-1 strains, and is now designated fusin (9). Fusin is not known to be a  $\beta$ -chemokine receptor (7-9).

To study how  $\beta$ -chemokines inhibit HIV-1 replication, a virus entry assay based on single-cycle infection by an env-deficient virus, NL4/3 $\Delta$ env (which also carries the luciferase reporter gene), complemented by envelope glycoproteins expressed in trans was used (10,11). Various env-complemented viruses were tested in PM1 cells, a variant of HUT-78 that has the unique ability to support replication of primary and TCLA HIV-1 strains, allowing comparison of envelope glycoprotein functions against a common cellular background (2,12). MIP-1 $\alpha$ , MIP-1 $\beta$  and RANTES are most active against HIV-1 in combination (2,3), and strongly inhibited infection of PM1 cells by complemented viruses whose envelopes are derived from the NSI primary strains ADA and BaL (Table 1a).

TABLE 1

Inhibition of HIV-1 entry in PM1 cells and CD4 <sup>+</sup> T-cells by $\beta$ -chemokines					
	% luciferase activity				
	BaL	ADA	NL4/3	HxB2	MuLV
a) PM1 cells					
control without virus	2	2	2	5	3
control with virus	100	100	100	100	100
+R/M $\alpha$ /M $\beta$ (50/50/50)	2	3	92	117	100
+RANTES (100)	1	1	nd	nd	nd
+MIP-1 $\alpha$ (100)	54	54	nd	nd	nd
+MIP-1 $\beta$ (100)	1	6	nd	nd	nd
+MCP-1 (100)	46	50	nd	nd	nd
+MCP-2 (100)	28	26	nd	nd	nd
+MCP-3 (100)	58	46	nd	nd	nd
b) LW4 CD4 <sup>+</sup> T-cells					
control without virus	1	1	1		
control with virus	100	100	100		
+R/M $\alpha$ /M $\beta$ (200/200/200)	14	68	nd		
LW5 CD4 <sup>+</sup> T-cells					
control without virus	1	1	1		
control with virus	100	100	100		
+R/M $\alpha$ /M $\beta$ (200/200/200)	15	73	nd		

Table 1 legend:

PM1 cells were cultured as described by Lusso et al (12). Ficoll/hypaque-isolated PBMC from laboratory workers (LW) stimulated with PHA for 72h before depletion of CD8<sup>+</sup> Lymphocytes with anti-CD8 immunomagnetic beads (DYNAL, Great Neck, N.Y.). CD4<sup>+</sup> Lymphocytes were maintained in culture medium containing interleukin-2 (100U/ml; Hoffmann LaRoche, Nutley, NJ.), as described previously (3). Target cells ( $1-2 \times 10^5$ ) were infected with supernatants (10-50 ng of HIV-1 p24) from 293-cells co-transfected with an NL4/3 $\Delta$ env-luciferase vector and a HIV-1 env-expressing vector (10,11).  $\beta$ -Chemokines (R & D Systems, Minneapolis) were added to the target cells simultaneously with virus, at the final concentrations (ng/ml) indicated in parentheses in the first column. The  $\beta$ -chemokine concentration range was selected based on prior studies (2,3). After 2 h, the cells were washed twice with PBS, resuspended in  $\beta$ -chemokine-containing media and maintained for 48-96 h. Luciferase activity in cell lysates was measured as described previously (10, 11). The values indicated represent luciferase activity (cpm)/ng p24/mg protein, expressed relative to that in virus-control cultures lacking  $\beta$ -chemokines (100%), and are the means of duplicate or sextuplicate determinations. nd, not done. R/M $\alpha$ /M $\beta$ , RANTES+ MIP-1 $\alpha$ +MIP-1 $\beta$ .

RANTES and MIP-1 $\beta$  were strongly active when added individually, while other  $\beta$ -chemokines—MIP-1 $\alpha$ , MCP-1, MCP-2 and MCP-3 (refs. 13-15)—were weaker inhibitors (Table 1a). However, MIP-1 $\alpha$ , MIP-1 $\beta$  and RANTES, in combination, did not inhibit infection of PM1 cells by the TCLA strains NL4/3 and HxB2, or by the amphotropic murine leukemia virus (MuLV-Ampho) pseudotype (Table 1a). Thus, phenotypic characteristics of the HIV-1 envelope glycoproteins influence their sensitivity to  $\beta$ -chemokines in a virus entry assay.

The env-complementation assay was used to assess HIV-1 entry into CD4<sup>+</sup> T-cells from two control individuals (LW4 and LW5). MIP-1 $\alpha$ , MIP-1 $\beta$  and RANTES strongly inhibited infection by the NSI primary strain JR-FL infection of LW4's and LW5's CD4<sup>+</sup> T-cells, and weakly reduced HxB2 infection of LW cells (Table 1b), suggesting that there may be some overlap in receptor usage on activated CD4<sup>+</sup> T-cells by different virus strains. BaL env-mediated replication in normal PBL was also inhibited by MIP-1 $\alpha$ , MIP-1 $\beta$  and RANTES, albeit with significant inter-donor variation in sensitivity (data not shown).

It was determined when  $\beta$ -chemokines inhibited HIV-1 replication by showing that complete inhibition of infection of PM1 cells required the continuous presence of  $\beta$ -chemokines for up to 5 h after addition of ADA or BaL env-complemented virus (FIG. 3a). Pre-treatment of the cells with  $\beta$ -chemokines for 2 h or 24 h prior to infection had no inhibitory effect if the cells were subsequently washed before virus addition. Furthermore, adding  $\beta$ -chemokines 2 h after virus only minimally affected virus entry (FIG. 3a). A PCR-based assay was next used to detect HIV-1 early DNA reverse transcripts in PM1 cells after 10 h of infection; reverse transcription of ADA, but not of NL4/3, could not be detected in the presence of MIP-1 $\beta$  and RANTES (FIG. 3b). Thus, inhibition by  $\beta$ -chemokines requires their presence during at least one of the early stages of HIV-1 replication: virus attachment, fusion and early reverse transcription.

As described in part in the First Series of Experiments, these sites of action were discriminated, first by testing whether  $\beta$ -chemokines inhibited binding of JR-FL or BRU (LAI) gp120 to soluble CD4, or of tetrameric CD4-IgG2 binding to HeLa-JR-FL cells expressing oligomeric envelope glycoproteins (17). No inhibition by any of the

$\beta$ -chemokines was found in either assay, whereas the OKT4a CD4-MAb was strongly inhibitory in both (FIG. 2 and data not shown). Thus,  $\beta$ -chemokines inhibit a step after CD4 binding, when conformational changes in the envelope glycoproteins lead to fusion of the viral and cellular membranes (18). Cell-cell membrane fusion is also induced by the gp120-CD4 interaction, and can be monitored directly by resonance energy transfer (RET) between fluorescent dyes incorporated into cell membranes (17). In the RET assay, OKT4a completely inhibits membrane fusion of PM1 cells with HeLa cells expressing the envelope glycoproteins of either JR-FL (HeLa-JR-FL, the same cell line referred to above as HeLa-env<sub>JR-FL</sub>) or BRU (HeLa-BRU, the same cell line referred to above as HeLa-env<sub>BRU</sub>), confirming the specificity of the process (17). RANTES, MIP-1 $\beta$  (and to a lesser extent, MIP-1 $\alpha$ ) strongly inhibited membrane fusion of HeLa-JR-FL cells with PM1 cells, whereas fusion between PM1 cells and HeLa-BRU cells was insensitive to these  $\beta$ -chemokines (FIG. 1 and Table 2a).

TABLE 2

Effect of $\beta$ -chemokines on HIV-1 envelope glycoprotein-mediated membrane fusion measured using the RET assay		
	% Fusion	
	HeLa-JR-FL	HeLa-BRU
<b>a) PM1 cells</b>		
no chemokines	100	100
+R/M $\alpha$ /M $\beta$ (80/400/100)	1	95
+RANTES (80)	8	100
+MIP-1 $\alpha$ (400)	39	100
+MIP-1 $\beta$ (100)	13	93
+MCP-1 (100)	99	98
+MCP-2 (100)	72	93
+MCP-3 (100)	98	99
<b>b) LW5 CD4<sup>+</sup> cells</b>		
no chemokines	100	100
+R/M $\alpha$ /M $\beta$ (106/533/133)	39	100
+RANTES (106)	65	95
+MIP-1 $\alpha$ (533)	72	100
+MIP-1 $\beta$ (133)	44	92
+OKT4A (3 $\mu$ g/ml)	0	0

Table 2 legend:

CD4<sup>+</sup> target cells (mitogen-activated CD4<sup>+</sup> lymphocytes or PM1 cells) were labeled with octadecyl rhodamine (Molecular Probes, Eugene, Oreg.), and HeLa-JR-FL cells,

HeLa-BRU cells (or control HeLa cells, not shown) were labeled with octadecyl fluorescein (Molecular Probes), overnight at 37° C. Equal numbers of labeled target cells and env-expressing cells were mixed in 96-well plates and  $\beta$ -chemokines (or CD4 MAb OKT4a) were added at the final concentrations (ng/ml) indicated in parentheses in the first column. Fluorescence emission values were determined 4 h after cell mixing (17). If cell fusion occurs, the dyes are closely associated in the conjoined membrane such that excitation of fluorescein at 450 nm results in resonance energy transfer (RET) and emission by rhodamine at 590nm. Percentage fusion is defined as equal to 100x[(Exp RET-Min RET)/(Max RET-Min RET)], where Max RET=% RET obtained when HeLa-Env and CD4<sup>+</sup> cells are mixed, Exp RET=% RET obtained when HeLa-Env and CD4<sup>+</sup> cells are mixed in the presence of fusion-inhibitory compounds, and Min RET=% RET obtained when HeLa cells (lacking HIV-1 envelope glycoproteins) and CD4<sup>+</sup> cells are mixed. The % RET value is defined by a calculation described elsewhere (17), and each is the mean of triplicate determinations. These values were, for HeLa-JR-FL and HeLa-BRU cells respectively: PM1 cells 11.5%, 10.5%; LW5 CD4<sup>+</sup> cells, 6.0%, 10.5%; R/M $\alpha$ /M $\beta$ , RANTES+MIP-1 $\alpha$ +MIP-1 $\beta$ .

Similar results were obtained with primary CD4<sup>+</sup> T-cells from LW5 (Table 2b), although higher concentrations of  $\beta$ -chemokines were required to inhibit membrane fusion in the primary cells than in PM1 cells. Thus, the actions of the  $\beta$ -chemokines are not restricted to the PM1 cell line. The RET assay demonstrates that  $\beta$ -chemokines interfere with env-mediated membrane fusion.

The simplest explanation of these results is that the binding of certain  $\beta$ -chemokines to their receptor(s) prevents, directly or otherwise, the fusion of HIV-1 with CD4<sup>+</sup> T-cells. It has been known for a decade that HIV-1 requires a second receptor for entry into CD4<sup>+</sup> cells (19-21). This function is supplied, for TCLA strains, by fusin (9). Several receptors for MIP-1 $\alpha$ , MIP-1 $\beta$  and RANTES have been identified (6,7), and  $\beta$ -chemokines exhibit considerable cross-reactivity in receptor usage (4-8). However, C-C CKR-1 and, especially, C-C CKR-5 were identified as the most likely candidates, based on tissue expression patterns and their abilities to bind MIP-1 $\alpha$ , MIP-1 $\beta$  and RANTES (4,7,8,15,22). C-C CKR-1, C-C CKR-5 and LESTR are each expressed at the mRNA level in PM1 cells and primary macrophages (data not shown). These and other  $\beta$ -chemokine receptors were therefore PCR-amplified, cloned and expressed.

The expression of C-C CKR-5 in HeLa-CD4 (human), COS-CD4 (simian) and 3T3-CD4 (murine) cells rendered each of them readily infectible by the primary, NSI strains ADA and BaL in the env-complementation assay of HIV-1 entry (Table 3).

TABLE 3

C-C CKR-5 expression permits infection of CD4-expressing cells by primary, NSI HIV-1 strains

		pcDNA3.1	LESTR	CKR-1	CKR-2a	CKR-3	CKR-4	CKR-5	R/M $\alpha$ /M $\beta$ CKR-5
COS-CD4	ADA	798	456	600	816	516	534	153000	3210
	BaL	660	378	600	636	516	618	58800	756
	HxB2	5800	96700	5240	5070	5470	5620	4850	5000
HeLa-CD4	ADA	678	558	4500	912	558	600	310000	6336
	BaL	630	738	1800	654	516	636	104000	750
	HxB2	337000	nd	nd	nd	nd	nd	nd	356000
3T3-CD4	ADA	468	558	450	618	534	606	28400	1220
	BaL	606	738	660	738	534	558	11700	756
	HxB2	456	24800	618	672	732	606	618	606

Table 3 legend:

Chemokine receptor genes C—C CKR-1, C—C CKR-2a, C—C CKR-3, C—C CKR-4 and C—C CKR-5 have no introns (4-8,15,22) and were isolated by PCR performed directly on a human genomic DNA pool derived from the PBMC of seven healthy donors. Oligonucleotides overlapping the ATG and the stop codons and containing BamHI and XhoI restriction sites for directional cloning into the pcDNA3.1 expression vector (Invitrogen Inc.) were used. LESTR (also known as fusin or HUMSTR) (4,9,24) was cloned by PCR performed directly on cDNA derived from PM1 cells, using sequences derived from the NIH database. Listed below are the 5' and 3' primer pairs used in first (5-1 and 3-1) and second (5-2 and 3-2) round PCR amplification of the CKR genes directly from human genomic DNA, and of LESTR from PM1 cDNA. Only a single set of primers was used to amplify CKR-5.

LESTR: L/5-1=AAG CTT GGAGAACCGG GTTACC  
ATG GAG GGG ATC (SEQ ID NO: 6);

L/5-2=GTC TGA GTC TGA GTC AAG CTT GGA GAA  
CCA (SEQ ID NO: 7);

L/3-1=CTC GAG CAT CTG TGT TAG CTG GAG TGA  
AAA CTT GAA GAC TC (SEQ ID NO: 8);

L/3-2=GTC TGA GTC TGA GTC CTC GAG CAT CTG  
TGT (SEQ ID NO: 9);

CKR-1: C1/5-1=AAG CTT CAG AGA GAA GCC GGG  
ATG GAA ACT CC (SEQ ID NO: 10);

C1/5-2=GTC TGA GTC TGA GTC AAG CTT CAG AGA  
GAA (SEQ ID NO: 11);

C1/3-1=CTC GAG CTG AGT CAG AAC CCA GCA GAG  
AGT TC (SEQ ID NO: 12);

C1/3-2=GTC TGA GTC TGA GTC CTC GAG CTG AGT  
CAG (SEQ ID NO: 13);

CKR-2a: C2/5-1=AAG CTT CAG TAC ATC CAC AAC  
ATG CTG TCC AC (SEQ ID NO: 14);

C2/5-2=GTC TGA GTC TGA GTC AAG CTT CAG TAC  
ATC (SEQ ID NO: 15);

C2/3-1=CTC GAG CCT CGT TTT ATA AAC CAG CCG  
AGA C (SEQ ID NO: 16);

C2/3-2=GTC TGA GTC TGA GTC CTC GAG CCT CGT  
TTT (SEQ ID NO: 17);

CKR-3: C3/5-1=AAG CTT CAG GGA GAA GTG AAA  
TGA CAA CC (SEQ ID NO: 18);

C3/5-2=GTC TGA GTC TGA GTC AAG CTT CAG GGA  
GAA (SEQ ID NO: 19);

C3/3-1=CTC GAG CAG ACC TAA AAC ACA ATA GAG  
AGT TCC (SEQ ID NO: 20);

C3/3-2=GTC TGA GTC TGA GTC CTC GAG CAG ACC  
TAA (SEQ ID NO: 21);

CKR-4: C4/5-1=AAG CTT CTG TAG AGT TAAAAATG  
AAC CCC ACG G (SEQ ID NO: 22);

C4/5-2=GTC TGA GTC TGA GTC AAG CTT CTG TAG  
AGT (SEQ ID NO: 23);

C4/3-1=CTC GAG CCA TTT CAT TTT TCT ACA GGA  
CAG CAT C (SEQ ID NO: 24);

C4/3-2=GTC TGA GTC TGA GTC CTC GAG CCA TTT  
CAT (SEQ ID NO: 25);

CKR-5: C5/5-12=GTC TGA GTC TGA GTC AAG CTT  
AAC AAG ATG GAT TAT CAA (SEQ ID NO: 26);

C5/3-12=GTC TGA GTC TGA GTC CTC GAG TCC GTG  
TCA CAA GCC CAC (SEQ ID NO: 27);

The human CD4-expressing cell lines HeLa-CD4 (P42), 3T3-CD4 (sc6) and COS-CD4 (Z28T1) (23) were transfected with the different pcDNA3.1-CKR constructs by the calcium phosphate method, then infected 48h later with different reporter viruses (200 ng of HIV-1 p24/10<sup>6</sup> cells) in the presence or absence of  $\beta$ -chemokines (400 ng/ml each of RANTES, MIP-1 $\alpha$  and MIP-1 $\beta$ ). Luciferase activity in cell

lysates was measured 48 h later (10,11).  $\beta$ -Chemokine blocking data is only shown for C—C CKR-5, as infection mediated by the other C—C CKR genes was too weak for inhibition to be quantifiable. In PCR-based assays of HIV-1 entry, a low level of entry of NL4/3 and ADA into C—C CKR-1 expressing cells (data not shown) was consistently observed.

Neither LESTR nor C—C CKR-1, -2a, -3 or -4 could substitute for C—C CKR-5 in this assay. The expression of LESTR in COS-CD4 and 3T3-CD4 cells permitted HxB2 entry, and HxB2 readily entered untransfected (or control plasmid-transfected) HeLa-CD4 cells (Table 3). Entry of BAL and ADA into all three C—C CKR-5-expressing cell lines was almost completely inhibited by the combination of MIP-1 $\alpha$ , MIP-1 $\beta$  and RANTES, whereas HxB2 entry into LESTR-expressing cells was insensitive to  $\beta$  chemokines (Table 3). These results suggest that C—C CKR-5 functions as a  $\beta$ -chemokine-sensitive second receptor for primary, NSI HIV-1 strains.

The second receptor function of C—C CKR-5 was confirmed in assays of env-mediated membrane fusion. When C—C CKR-5 was transiently expressed in COS and HeLa cell lines that permanently expressed human CD4, both cell lines fused strongly with HeLa cells expressing the JR-FL envelope glycoproteins, whereas no fusion occurred when control plasmids were used (data not shown). Expression of LESTR instead of C—C CKR-5 did not permit either COS-CD4 or HeLa-CD4 cells to fuse with HeLa-JR-FL cells, but did allow fusion between COS-CD4 cells and HeLa-BRU cells (data not shown).

The fusion capacity of S-chemokine receptors was also tested in the RET assay. The expression of C—C CKR-5, but not of C—C CKR-1, -2a, -3 or -4, permitted strong fusion between HeLa-CD4 cells and HeLa-JR-FL cells. The extent of fusion between HeLa-JR-FL cells and C—C CKR-5-expressing HeLa-CD4 cells was greater than the constitutive level of fusion between HeLa-BRU cells and HeLa-CD4 cells (FIG. 4). The fusion-conferring function of C—C CKR-5 for primary, NSI HIV-1 strains has therefore been confirmed in two independent fusion assays.

#### Experimental Discussion

Together, the above results establish that MIP-1 $\alpha$ , MIP-1 $\beta$  and RANTES inhibit HIV-1 infection at the entry stage, by interfering with the virus-cell fusion reaction subsequent to CD4 binding. It was also shown that C—C CKR-5 can serve as a second receptor for entry of primary NSI strains of HIV-1 into CD4<sup>+</sup> T-cells, and that the interaction of  $\beta$ -chemokines with C—C CKR-5 inhibits the HIV-1 fusion reaction.

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#### Third Series of Experiments

The chemokine SDF-1 (stromal cell-derived factor 1) is the natural ligand for Fusin/CXCR4 and blocks infection by laboratory-adapted strains of HIV-1 (Ref. 1 and 2). SDF-1 exists as at least two forms, SDF-1 $\alpha$  and SDF-1 $\beta$  based on variable splicing of the SDF-1 gene (Ref. 1 and 3). In the RET assay, this chemokine specifically inhibits membrane fusion mediated by gp120/gp41 from the laboratory-adapted strain HIV-1<sub>LAI</sub> but not by gp120/gp41 from the macrophage-tropic isolate HIV-1<sub>JR-FL</sub> as shown in FIG. 5.

#### References of the Third Series of Experiments

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#### Fourth Series of Experiments

##### Direct Binding of HIV-1<sub>JR-FL</sub> gp120 to CCR5<sup>+</sup> CD4<sup>+</sup> Cells

The direct binding of HIV-1<sub>JR-FL</sub> gp120 to CCR5<sup>+</sup> CD4<sup>+</sup> cells has been demonstrated. In this case, preincubation of the gp120 with sCD4 or another CD4-based molecule is required, presumably because this results in a conformational change in gp120 that exposes a chemokine receptor binding site. FIG. 6 illustrates the use of flow cytometry to measure the direct binding of sCD4/gp120 complexes to human CCR5-bearing murine L1.2 cells. Background levels of binding were observed with either biotinylated protein alone, or if gp120 from the laboratory-adapted strain HIV-1<sub>LAI</sub> is used in place of the HIV-1<sub>JR-FL</sub> gp120 (data not shown).

This assay has been adapted for drug screening purposes to a 96-well microplate format where binding of the sCD4/gp120 complexes to CCR5<sup>+</sup>/CD4<sup>+</sup> cells is measured using a fluorometric plate reader. One method is as follows:

- 1) Plate out L1.2-CCR5<sup>+</sup> cells (approx. 500,000/well).
- 2) Add inhibitor for 1 hour at room temperature.
- 3) Wash and add biotinylated sCD4 (2.5  $\mu$ g/ml) and biotinylated HIV-1<sub>JR-FL</sub> gp120 (5  $\mu$ g/ml), then incubate for 2 hours at room temperature.
- 4) Wash and incubate with streptavidin-phycoerythrin (100 ng/ml).
- 5) Wash and measure the amount of bound gp120/sCD4 using a fluorometric plate reader exciting at 530 nm and reading emission at 590 nm.

Using this method, inhibition of binding of gp120/sCD4 to CCR5 by CC-chemokines (FIG. 7) and antibodies to CCR5 that block HIV-1 infection (not shown) have been demonstrated.

##### Inhibition of HIV-1 envelope-mediated membrane fusion by the bicyclam, JM3100.

The bicyclam JM3100, obtained from Dr. J. Moore (Aaron Diamond AIDS Research Center, NY) was tested for ability to inhibit membrane fusion mediated by the envelope glycoproteins of the LAI or JR-FL strains of HIV-1 using the resonance energy transfer (RET) assay described above. As illustrated in FIG. 7, this molecule specifically and potently inhibits fusion mediated by gp120/gp41 from the HIV-1<sub>LAI</sub> strain, and not from the HIV-1<sub>JR-FL</sub> strain. These data suggest that this molecule specifically inhibits HIV fusion by blocking the interaction between HIV-1<sub>LAI</sub> gp120 and CXCR4.

#### SEQUENCE LISTING

##### (1) GENERAL INFORMATION:

(iii) NUMBER OF SEQUENCES: 27

##### (2) INFORMATION FOR SEQ ID NO:1:

###### (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 38 nucleotides
- (B) TYPE: nucleic acid

-continued

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(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CAAGGCTACT TCCCTGATTG CGAAGACTAC ACACCAGG 38

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 25 nucleotides  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

AGCAAGCCGA GTCCTGCGTC GAGAG 25

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 23 nucleotides  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GGGACTTTCC GCTGGGGACT TTC 23

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 33 nucleotides  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CCTGTTCGGG CGCCACTGCT AGAGATTTTC CAC 33

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 60 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: Not Relevant  
(D) TOPOLOGY: Not Relevant

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Pro Cys Cys Phe Ala Tyr Ile Ala Arg Pro Leu Pro Arg Ala His Ile Lys  
1 5 10 15

Glu Tyr Phe Tyr Thr Ser Gly Lys Cys Ser Asn Pro Ala Val Val Phe Val  
20 25 30

Thr Arg Lys Asn Arg Gln Val Cys Ala Asn Pro Glu Lys Lys Trp Val Arg  
35 40 45 50

Glu Tyr Ile Asn Ser Leu Glu Met Ser  
55 60

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## (2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 36 nucleotides  
    (B) TYPE: nucleic acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

AAGCTTGGAG AACCAGCGGT TACCATGGAG GGGATC

36

## (2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 30 nucleotides  
    (B) TYPE: nucleic acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GTCTGAGTCT GAGTCAAGCT TGGAGAACCA

30

## (2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 41 nucleotides  
    (B) TYPE: nucleic acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CTCGAGCATC TGTGTTAGCT GGAGTGAAA CTTGAAGACT C

41

## (2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 30 nucleotides  
    (B) TYPE: nucleic acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GTCTGAGTCT GAGTCCTCGA GCATCTGTGT

30

## (2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 32 nucleotides  
    (B) TYPE: nucleic acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

AAGCTTCAGA GAGAAGCCGG GATGGAACT CC

32

## (2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:

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(A) LENGTH: 30 nucleotides  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GTCTGAGTCT GAGTCAAGCT TCAGAGAGAA 30

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 32 nucleotides  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CTCGAGCTGA GTCAGAACCC AGCAGAGAGT TC 32

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 30 nucleotides  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GTCTGAGTCT GAGTCCTCGA GCTGAGTCAG 30

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 32 nucleotides  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

AAGCTTCAGT ACATCCACAA CATGCTGTCC AC 32

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 30 nucleotides  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GTCTGAGTCT GAGTCAAGCT TCAGTACATC 30

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 31 nucleotides  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CTCGAGCCTC GTTTTATAAA CCAGCCGAGA C

31

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 nucleotides
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GTCTGAGTCT GAGTCCTCGA GCCTCGTTTT

30

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 29 nucleotides
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

AAGCTTCAGG GAGAAGTGAA ATGACAACC

29

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 nucleotides
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GTCTGAGTCT GAGTCAAGCT TCAGGGAGAA

30

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 nucleotides
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

CTCGAGCAGA CCTAAAACAC AATAGAGAGT TCC

33

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 nucleotides
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

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GTCTGAGTCT GAGTCCTCGA GCAGACCTAA

30

## (2) INFORMATION FOR SEQ ID NO:22:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 34 nucleotides
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

AAGCTTCTGT AGAGTTAAAA AATGAACCCC ACGG

34

## (2) INFORMATION FOR SEQ ID NO:23:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 30 nucleotides
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

GTCTGAGTCT GAGTCAAGCT TCTGTAGAGT

30

## (2) INFORMATION FOR SEQ ID NO:24:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 34 nucleotides
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

CTCGAGCCAT TTCATTTTTC TACAGGACAG CATC

34

## (2) INFORMATION FOR SEQ ID NO:25:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 30 nucleotides
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

GTCTGAGTCT GAGTCCTCGA GCCATTTCAT

30

## (2) INFORMATION FOR SEQ ID NO:26:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 39 nucleotides
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

GTCTGAGTCT GAGTCAAGCT TAACAAGATG GATTATCAA

39

-continued

## (2) INFORMATION FOR SEQ ID NO:27:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 39 nucleotides
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotides

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

GTCTGAGTCT GAGTCTCGA GTCCGTGTCA CAAGCCAC

39

15

What is claimed is:

1. An in vitro method for determining whether an agent is capable of inhibiting HIV-1 infection of a CD4<sup>+</sup> cell susceptible to HIV-1 infection comprising the steps of:

- (a) fixing a chemokine receptor on a solid matrix wherein the chemokine receptor is a co-receptor for HIV-1 infection;
- (b) contacting the fixed chemokine receptor with the agent under conditions permitting binding of the agent to the chemokine receptor;
- (c) removing any unbound agent;
- (d) contacting the resulting fixed chemokine receptor to which the agent is bound with a predetermined amount of gp120/CD4<sup>+</sup> complex under conditions permitting binding of gp120/CD4<sup>+</sup> complex to the fixed chemokine receptor in the absence of the agent;
- (e) removing any unbound gp120/CD4<sup>+</sup> complex;
- (f) measuring the amount of gp120/CD4<sup>+</sup> complex bound to the fixed chemokine receptor; and
- (g) comparing the amount measured in step (f) with the amount measured in the absence of the agent, a decrease in the amount bound in the presence of the agent indicating that the agent is capable of inhibiting HIV-1 infection.

2. An in vitro method for determining whether an agent is capable of inhibiting HIV-1 infection of a CD4<sup>+</sup> cell susceptible to HIV-1 infection comprising the steps:

- (a) fixing a chemokine receptor on a solid matrix wherein the chemokine receptor is a co-receptor for HIV-1 infection;
- (b) contacting the fixed chemokine receptor with the agent and a predetermined amount of gp120/CD4<sup>+</sup> complex under conditions permitting binding of the gp120/CD4<sup>+</sup> complex to the fixed chemokine receptor in the absence of the agent;
- (c) removing any unbound agent or unbound gp120/CD4<sup>+</sup> complex or both;
- (d) measuring the amount of gp120/CD4<sup>+</sup> complex bound to the fixed chemokine receptor; and
- (e) comparing the amount measured in step (d) with the amount measured in the absence of the agent, a decrease in the amount bound in the presence of the agent indicating that the agent is capable of inhibiting HIV-1 infection.

3. An in vitro method for determining whether an agent is capable of inhibiting HIV-1 infection of a CD4<sup>+</sup> cell susceptible to HIV-1 infection comprising steps of:

- (a) fixing a gp120/CD4<sup>+</sup> complex on a solid matrix;
- (b) contacting the fixed gp120/CD4<sup>+</sup> complex with the agent under conditions permitting the binding of the agent to the gp120/CD4<sup>+</sup> complex;

(c) removing any unbound agent;

(d) contacting the resulting fixed gp120/CD4<sup>+</sup> complex to which the agent is bound with a predetermined amount of chemokine receptor, wherein the chemokine receptor is a co-receptor for HIV-1 infection, under conditions permitting binding of the chemokine receptor to the fixed gp120/CD4<sup>+</sup> complex in the absence of the agent;

(e) removing any unbound chemokine receptor;

(f) measuring the amount of chemokine receptor bound to the fixed gp120/CD4<sup>+</sup>; and

(g) comparing the amount measured in step (f) with the amount measured in the absence of the agent, a decrease in the amount bound in the presence of the agent indicating that the agent is capable of inhibiting HIV-1 infection.

4. An in vitro method for determining whether an agent is capable of inhibiting HIV-1 infection of a CD4<sup>+</sup> cell susceptible to HIV-1 infection comprising steps of:

- (a) fixing a gp120/CD4<sup>+</sup> complex on a solid matrix;
- (b) contacting the fixed gp120/CD4<sup>+</sup> complex with the agent and a predetermined amount of chemokine receptor, wherein the chemokine receptor is a co-receptor for HIV-1 infection, under conditions permitting binding of the chemokine receptor to the fixed gp120/CD4<sup>+</sup> complex in the absence of the agent;
- (c) removing any unbound agent or any unbound chemokine receptor or both;
- (d) measuring the amount of chemokine receptor bound to the fixed gp120/CD4<sup>+</sup>; and
- (e) comparing the amount measured in step (d) with the amount measured in the absence of the agent, a decrease in the amount bound in the presence of the agent indicating that the agent is capable of inhibiting HIV-1 infection.

5. The method of claim 1, 2, 3, or 4 wherein the CD4<sup>+</sup> is a soluble CD4<sup>+</sup>.

6. The method of claim 1, 2, 3, or 4 wherein the chemokine receptor is expressed on a cell.

7. The method of claim 6 wherein the cell is a L1.2 cell.

8. The method of claim 1 or 2, wherein the gp120, CD4<sup>+</sup> or both are labeled with a detectable marker.

9. The method of claim 3 or 4 wherein the chemokine receptor is labeled with a detectable marker.

10. The method of claim 1 or 2, wherein the gp120, CD4<sup>+</sup> or both are labeled with biotin.

11. The method of claim 2 or 4 wherein the chemokine receptor is labeled with biotin.

12. The method of any one of claims 1, 2, 3, or 4, wherein the chemokine receptor is CCR5.

\* \* \* \* \*



US005817458A

**United States Patent** [19]**King et al.**[11] **Patent Number:** **5,817,458**[45] **Date of Patent:** **Oct. 6, 1998****[54] REAGENT SYSTEM FOR DETECTING HIV-  
INFECTED PERIPHERAL BLOOD  
LYMPHOCYTES IN WHOLE BLOOD****[75] Inventors:** **Chester F. King, Frederick; Robert A.  
Hallowitz, Gaithersburg, both of Md.****[73] Assignee:** **The Avriel Group, AMCAS Division  
Inc.; a part interest****[21] Appl. No.:** **732,782****[22] Filed:** **Oct. 15, 1996****[51] Int. Cl.<sup>6</sup>** ..... **C12Q 1/70; A01N 1/02;  
G01N 33/53; G01N 33/567****[52] U.S. Cl.** ..... **435/5; 435/2; 435/721;  
436/512; 436/513; 436/518; 436/526; 436/531****[58] Field of Search** ..... **435/2, 5, 7.21,  
435/7.32, 239, 803; 436/512, 513, 518,  
531, 526****[56] References Cited****U.S. PATENT DOCUMENTS**

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component of the receptor for the AIDS retrovirus", *Nature*,  
vol. 312 (20/27), pp. 763767, Dec. 1984.****Primary Examiner—Lynette F. Smith****Assistant Examiner—Brett Nelson****Attorney, Agent, or Firm—Millen, White, Zelano &  
Branigan, P.C.****[57]****ABSTRACT**

Fluorometric immunological assay method for detection of HIV-1 infection in which Murine anti-gp120 monoclonal antibodies coupled to paramagnetic microspheres (14) and Fluorescein conjugated anti-gp120 polyclonal antibodies IgG (16) are incubated in a few drops of whole blood diluted in 0.5 cc phosphate buffered saline (10). After incubation for 5 minutes, the HIV-infected peripheral blood lymphocytes (18) will be coated with both the Murine anti-gp120 monoclonal antibodies coupled to paramagnetic microspheres (14) and Fluorescein conjugated anti-gp120 polyclonal antibodies IgG (16) at exposed gp120 antigens (20) binding sites. At the time of measurement said HIV- infected peripheral blood lymphocytes (18) will be pulled against the wall of the measurement vessel by means of a magnetic gradient (26). The cells adhering to the vessel wall are illuminated at 488 nm monochromatic light by a focused light source (28) and the resultant emitted fluorescence is imaged, measured and recorded.

**17 Claims, 2 Drawing Sheets**



FIG. 1

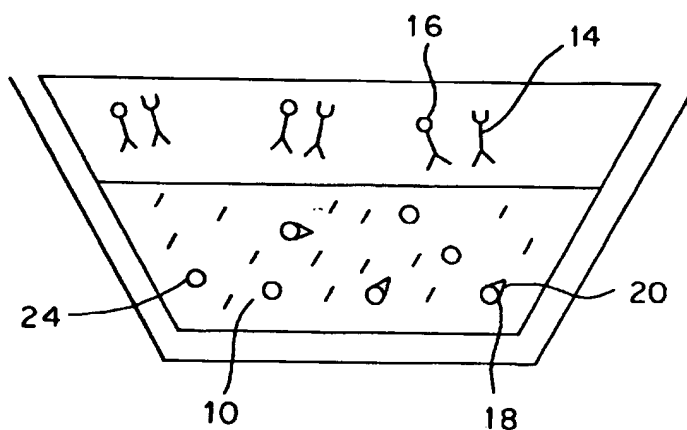


FIG. 2

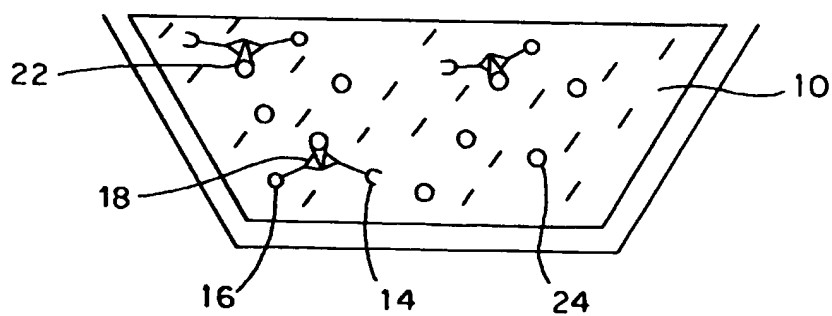


FIG. 3

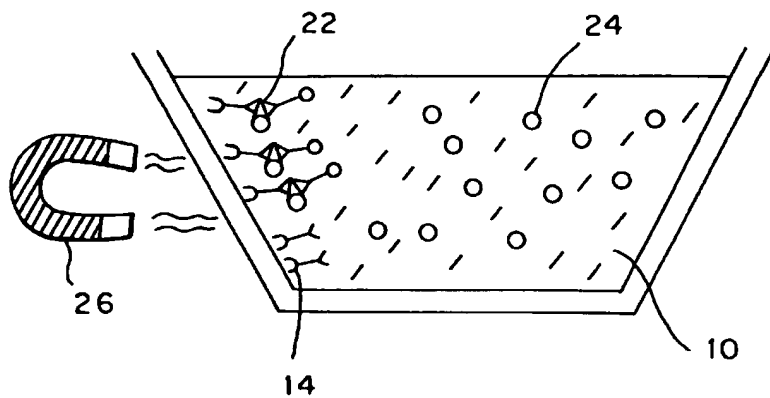
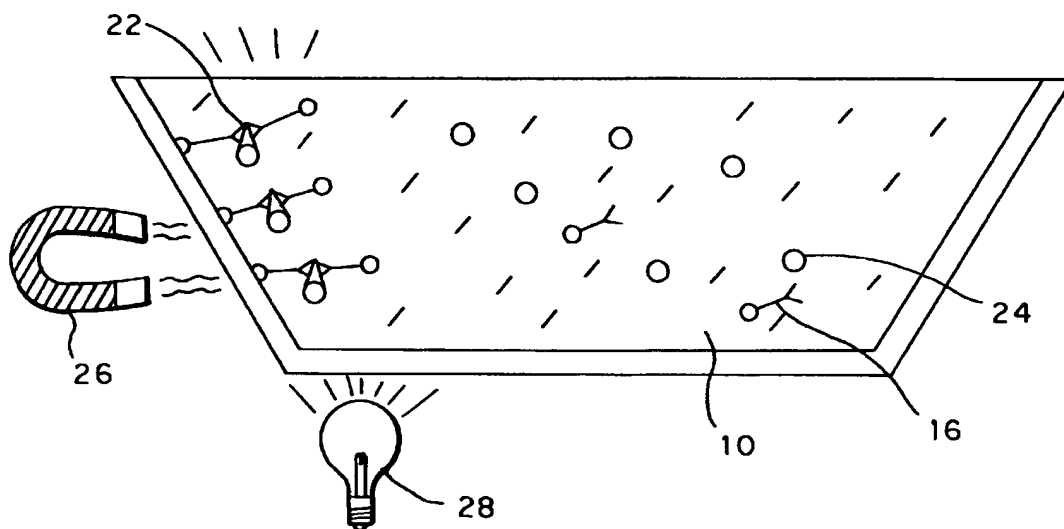


FIG. 4



# REAGENT SYSTEM FOR DETECTING HIV- INFECTED PERIPHERAL BLOOD LYMPHOCYTES IN WHOLE BLOOD

## CROSS REFERENCES TO RELATED APPLICATIONS

The invention is to be used with another invention known as a cartridge antigen test, which allows for the collection and mixing of blood with reagents in one package, and which can be viewed on a fluorescent microscope.

The invention is to be used with another invention known as the Mehica GP120 Dectector, an automated fluorescent microscope system that incubates and reads cartridge antigen tests.

### 1. Background—Field of Invention

The present invention relates to the early diagnosis of Human Immunodeficiency Virus (HIV) infections. More particularly, the invention provides compositions and methods for utilizing commercially available, high affinity and highly specific magnetically coupled monoclonal antibodies to the envelope surface glycoprotein of HIV-1 known as gp120, along with commercially available FITC conjugated polyclonal antibodies to gp120 for the purpose of isolating and fluorescing HIV-1 infected peripheral blood lymphocytes in whole blood.

### 2. Background—Description of Prior Art

The present invention relates generally to methods and materials useful in the early diagnosis of infection with HIV. More particularly, the invention provides compositions and methods for utilizing commercially available, high affinity and highly specific magnetically coupled monoclonal antibodies to the envelope surface glycoprotein of the HIV-1 known as gp120, along with commercially available FITC conjugated polyclonal antibodies to gp120 for the purpose of isolating and fluorescing HIV-1 infected peripheral blood lymphocytes in whole blood.

The state of the art with respect to the epidemiology and immunology of the causative agent of Auto-immune Deficiency Syndrome (AIDS) in humans is well summarized in: Laurence, "The Immune System and AIDS," *Scientific American*, 254, 12, 84-93 (1985); Gallo, "The First Human Retrovirus," *Scientific American*, 256, 12, 88-98, (1986); Gallo, "The AIDS Virus," *Scientific American*, 256, 1, 47-56 (1987); Levy, et al., *Science*, 225, 840-842 (1984); "Mobilizing against AIDS," Institute of Medicine, National Academy of Sciences, Harvard University Press (Cambridge, Mass., 1986); and Lane, et al., *Ann. Rev. Immunol.*, 3, 477-500 (1985).

The role of the CD4 surface glycoprotein of human T lymphocytes in infection by HIV has been extensively studied as represented by: Dalgeleish, et al., *Science*, 312, 763-767 (1984); Klatzmann, et al., *Science*, 225, 767-768 (1984); Klatzmann, et al., *Science*, 225, 59-62 (1984); McDoual, et al., *J. Immunol.*, 135, 3151-3162 (1985); and Maddon, et al., *Cell*, 47, 333-348 (1986).

Infection of a T cell with HIV-1 follows from interaction between an epitope borne by HIV-1 and the CD4 receptor which is located on the T cell surface. The epitope on HIV-1 is borne by the envelope glycoprotein gp120 (molecular weight 120 kilodaltons). The glycoprotein gp120 is structurally exposed on the outside of the HIV-1 envelope. The gp120 binds to the CD4 antigens which exist on the cell surface of the helper T cells, etc., and in addition to providing the fusion point between the virus and the T helper cell, gp120 possesses activity which results in syncytium

formation, the mechanism of cell to cell infection with HIV-1, as described in detail in U.S. Pat. No. 4,725,699.

In light of the above background information regarding HIV and AIDS, it can be deduced that antibodies specific for the envelope of the virus, which plays such an important role in the establishment of the viral infection, could have great significance in identifying the most crucial cell-bound antigens on the surface of infected cells in the peripheral blood.

A number of research groups have reported successful development of murine monoclonal antibody specific for gp120. For example, T. C. Can, et al. (*Eur. J. Immunol.* 16:1465, 1986) reported that they chemically synthesized a portion of the peptide chain of gp120 and then prepared monoclonal antibodies (mAbs) specific for the synthetic peptide. They employed those mAbs in an indirect fluorescent antibody technique and reported they were able to detect HIV infection with greater sensitivity than was possible with the reverse transcriptase determination technique. Additional reports of murine anti-gp120 mAbs have been reported by Gostling et al., *J. Clin. Microbiol.*, 25, 845 (1987) and Matsushida et al., *Medical Immunol.*, 14, 307, (1987).

The present invention is concerned with a fluoremetric immunoassay in which a pair of manufactured non-competitive antibodies to gp120 are utilized. One antibody (mAb) is coupled to paramagnetic particles, while the second is in conjugate with FITC. The present invention takes advantage of the technology of immunomagnetic separation developed over the past 15 years to enrich or separate out of a mixture of cells, specific cellular components based on their specific immunological markers. The prior art is exemplified by U.S. Pat. Nos. 4,77,145; 4,731,337; 5,186,827; 5,238,810; 5,279,936; 5,411,863; and 4,935,147.

In these inventions particular methods are disclosed for separating a substance from a liquid medium using magnetic particles. None of these inventions, however, are specific for the process of using immunomagnetic particles for the diagnosis of HIV in whole blood. The present invention relies upon the commercial availability of high affinity anti-gp120 mAbs coupled with magnetic particles and a second non-competitive anti-gp120 polyclonal antibody (pAb) conjugated with FITC to fluorimetrically "tag" an HIV-infected cell and then magnetically separate it from uninfected cells in whole blood.

Of particular importance to the background of the present invention is the consideration of factors that demonstrate the importance of creating a diagnostic system which takes advantage of the above described molecular biology of HIV infection. It is also important to understand the need for the present invention based upon the limitations posed by current screening and confirmatory test protocols which are still mainly dependent upon host immune response to HIV infection by antibody production.

Testing serum for antibodies to HIV is currently the most cost-effective and accurate method of screening for and confirmation of infection. References concerning this include: Centers for Disease Control, "Update: Serologic Testing for Antibody to Human Immunodeficiency Virus." *MMWR*, 36, 833-40 (1988); Schwartz, J. S., Dans, P. E., Kinoshian, B. P., "Human Immunodeficiency Virus Test Evaluation, Performance and Use." *JAMA*, 259, 2574-9 (1988); Burke, D. S., Brundage, J. F., Redfield, R. R., et al. "Measurement of the False Positive Rate in a Screening Program for Human Immunodeficiency Virus Infections." *New England Journal of Medicine*, 319, 961-4 (1988);

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HIV antibody tests have their limitations. Usually antibodies to HIV appear within 3-6 months and as early as 6-8 weeks after infection, but silent infections have been documented in which seroconversion has occurred as late as 3 years from the moment of exposure. Therefore, because an infected person does not develop antibodies immediately, a negative test result cannot rule out HIV infection.

It has been shown that the majority (90%) of people first testing positive for HIV will develop AIDS within one year. This strongly suggests that the average person identifying HIV infection has been positive for an average of 8-9 years, in view of the fact that the average interval between infection and AIDS is 9-10 years.

This is particularly problematic because of the behavioral studies indicating that a person practicing high risk behaviors is likely to seek testing within days or a few weeks of the high risk behavior. That person is then likely to forget about HIV and continue risky behavior based on the false reassurance of a negative test performed before seroconversion was even possible.

The consequences of the above observations are:

1. The majority of people practicing intermittent high risk behaviors tend to seek reassurances very shortly after committing such behaviors.
2. They get reassurance and false security because of false-negative tests based on as yet undetectable antibody levels.
3. The majority of infected people continue intermittent or continuous high risk behaviors for 8-9 years after becoming infected.
4. These people are, therefore, transmitting HIV for 8-9 years.
5. If affordable, accurate testing could be accomplished within the brief interval between risky behavior and seroconversion, a significant increase in early HIV detection would be likely.
6. Therefore, affordable early detection would create a significant reduction in the high prevalence of HIV transmission by the falsely assured and oblivious people in the 90% majority cited above.

In a study of consistent sequential detection of RNA, antigen and antibody in early HIV infection, sequential appearance in blood of HIV RNA, HIVag, and HIV antibody was found. Data derived from testing seroconversion panels demonstrate a consistent sequential rise in the concentrations of HIV RNA followed by HIV antigen (p24), followed by anti-HIV in early HIV infection. Based on the timing of the appearance of RNA and antigen it was concluded that HIV RNA and HIVag could be used to confirm early infection. RNA and/or HIVag tests were concluded to be potentially useful for earlier detection of HIV infection (e.g. blood screening). The results of this study were published by Busch, M., Schumacher, Richard T., Stramer, S., et al., "Consistent Sequential Detection of RNA, Antigen and Antibody in Early HIV Infection: Assessment of the Window Period" Irwin Memorial Blood Center, San Francisco,

Calif., Boston Biomedical, Inc., Bridgewater, Mass. Poster presented at XI International AIDS Conference, Vancouver, BC, July, 1996.

Efforts have been made to close this "window" between exposure and antibody detectability. The p24 antigen test has already been mandated for use by all registered blood and plasma centers because of a partial closure of the "window" achieved by this method. However, in the best-case scenario, p24 antigen detection realistically only closes the window by 6-7 days. Since a significant rate of viral reproduction occurs within the first week of infection causing the presence of the envelope glycoprotein gp120 bearing lymphocytes in the peripheral blood, detection of blood-bound gp120 is an effective means to close the "window" even further than the p24 test.

#### FURTHER DESCRIPTION OF THE RELATED ART

A method for determining the concentration of substances in biological fluids (e.g., drugs, hormones, vitamins and enzymes) wherein magnetically responsive, permeable, solid, water insoluble, microparticles are employed is disclosed in U.S. Pat. No. 4,115,534. Functional magnetic particles formed by dissolving a mucopolysaccharide such as chitosan in acidified aqueous solution containing a mixture of ferrous chloride and ferric chloride is disclosed in

U.S. Pat. No. 4,285,819. The microspheres may be employed to remove dissolved ions from waste aqueous streams by formation of chelates. U.S. Pat. No. 3,933,997 describes a solid phase radio immunoassay for digoxin where anti-digoxin antibodies are coupled to magnetically responsive particles.

Small magnetic particles coated with an antibody layer are used in U.S. Pat. No. 3,970,518 to provide a large and widely distributed surface area for sorting out and separating select organisms and cells from populations thereof. U.S. Pat. No. 4,018,886 discloses small magnetic particles used to provide a large and widely distributed surface area for separating a select protein from a solution to enable detection thereof. The particles are coated with a protein that will interact specifically with the select protein.

U.S. Pat. No. 4,070,246 describes compositions comprising stable, water insoluble coatings on substrates to which biologically active proteins can be covalently coupled so that the resulting product has the biological properties of the protein and the mechanical properties of the substrate, for example, magnetic properties of a metal support.

A diagnostic method employing a mixture of normally separable protein-coated particles is discussed in U.S. Pat. No. 4,115,535. Microspheres of acrolein homopolymers and copolymer(s) with hydrophilic comonomers such as methacrylic acid and/or hydroxyethylmethacrylate are discussed in U.S. Pat. No. 4,413,070. U.S. Pat. No. 4,452,774 discloses magnetic iron-dextran microspheres which can be covalently bonded to antibodies, enzymes and other biological molecules and used to label and separate cells and other biological particles and molecules by means of a magnetic field. Coated magnetizable microparticles, reversible suspensions thereof, and processes relating thereto are disclosed in U.S. Pat. No. 4,454,234. A method of separating cationic from anionic beads in mixed resin beds employing a ferromagnetic material intricately incorporated with each of the ionic beads is described in U.S. Pat. No. 4,523,996. A magnetic separation method utilizing a colloid of magnetic particles is discussed in U.S. Pat. No. 4,526,681. U.K. Patent Application GB No. 2,152,664A discloses magnetic assay reagents.

An electron-dense antibody conjugate made by the covalent bonding of an iron-dextran particle to an antibody molecule is reported by Dutton, et al., *Proc. Natl. Acad. Sci.*, 76, 3392-3396 (1979). Ithakissios, et al. describes the use of protein containing magnetic microparticles in radioassays in *Clin. Chem.*, 23, 2072-2079 (1977). The separation of cells labeled with immunospecific iron dextran microspheres using high gradient magnetic chromatography is disclosed by Molday, et al., *FEBS*, 170, 232-238 (1984). In *J. Immunol. Meth.*, 52, 353-367 (1982) Molday, et al. describe an immuno-specific ferromagnetic iron-dextran reagent for the labeling and magnetic separation of cells. An application of magnetic microspheres in labeling and separation of cells is also disclosed by Molday, et al. in *Nature*, 268, 437 (1977). A solid phase fluoroimmunoassay of human albumin and biological fluids is discussed by Margessi, et al., *Clin. Chim. Acta.*, 89, 455-460 (1978). Nye, et al., *Clin. Chim. Acta.*, 69, 387-396 (1976) disclose a solid phase magnetic particle radioimmunoassay. Magnetic fluids are described by Rosenweig, *Scien. Amer.*, 252, 10,136-194 (1983). Magnetic protein A microspheres and their use in a method for cell separation are disclosed by Widdler, et al., *Clin. Immunol. and Immunopath.*, 14,395-400 (1979).

U.S. Pat. No. 5,279,936 is a method directed to the separation of a component of interest from other components of a mixture by causing the binding of the component of interest to magnetic particles. In the embodiment of the invention which is a method to separate cells from a mixture containing other components, the method comprises layering a first liquid medium containing cells and other components with a second medium which is of a different density than and/or different viscosity than the first liquid medium. The cells are bound to paramagnetic particles. The layered first liquid medium and the second liquid medium are subjected to a magnetic field gradient to cause the cell particles to migrate into the second medium. The purpose of isolating the cells in the second liquid medium is then, by a further embodiment, to separate the cells from the second liquid medium.

In the current invention, there is no need for a second liquid medium because the magnetic separation of HIV-1 infected cells is accomplished in the medium of phosphate buffered saline (PBS) diluted blood, by bringing the infected cells to a predetermined point in the reaction vessel. The only task required after separation is the illumination of the point of highest magnetic field concentration, to ascertain the presence or absence of high density specific fluorescence, which if present would indicate the presence of fluorescently tagged HIV-infected peripheral blood lymphocytes.

U.S. Pat. No. 4,935,147 is a method that specifically targets the application of magnetic separation in the assay of organic and inorganic biochemical analytes, particularly those analytes of interest in the analysis of body fluids. The method of this invention provides a way of separating non-magnetic particles from a medium by virtue of the chemically controlled non-specific reversible binding of such particles to magnetic particles. Because of the small size of the magnetic particles, it also provides for a very rapid binding of a substance to be separated. By then aggregating the particles there is provided a much more rapid and complete magnetic separation than has been achieved by previous methods.

In the current invention, this technique of magnetic separation does not apply because of the fact the antigen of interest is bound to cells, and therefore not in solution or in need of agglutination for separation. The current invention

merely requires the adherence of the many magnetic particles to an infected cell surface to magnetically pull the entire cell of interest to a predetermined point in the reaction vessel for viewing.

## OBJECTS AND ADVANTAGES

Accordingly, several objects and advantages of our invention are to provide a cost-effective, accurate means of early (within 4 days of exposure) HIV-1 infection detection in whole blood that was based on the ability to immunochemically/magnetically isolate and fluorescently label HIV-1 infected peripheral blood lymphocytes.

The advantages of the invention are:

1. Cell-bound antigen-based test closes the window period created by having to rely on the host immune system to produce antibodies against HIV-1 antigens to around 4 days.
2. Multi-purpose cartridge and fully automated incubator, magnetic separator and imaging system permit operation by non-medically trained personnel.
3. Appearance of cell-bound gp120 parallels appearance of viral genetic material, enabling invention to detect HIV presence in same time period as the current industry standard, the polymerase chain reaction (PCR) test at a small fraction of the cost.
4. Functional design of the multi-purpose cartridge permits a complete, self-contained, disposable unit that is much easier to handle than the PCR test for viral genetic material.
5. Entire test procedure requires minutes to turn around compared with weeks for PCR test.
6. Cost per test will be in tens of dollars rather than hundreds. Still further objects and advantages will become apparent from a consideration of the ensuing description and accompanying drawings.

## BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows reagents unmixed with whole blood.

FIG. 2 shows reagents reacting with gp120 on surface of infected peripheral blood lymphocytes.

FIG. 3 shows magnetic field gradient separates tagged infected peripheral blood lymphocytes infected peripheral blood lymphocytes.

FIG. 4 shows separated infected fluorescent tagged peripheral blood lymphocytes illuminated and fluoresced by laser light.

## REFERENCE NUMERALS

- 10 Whole blood diluted in approximately 0.5 cc phosphate buffered saline (PBS)
- 14 Murine anti-gp120 monoclonal antibodies (mAbs) coupled to paramagnetic microspheres
- 16 Fluorescein conjugated anti-gp120 polyclonal antibodies (pAbs) IgG
- HIV-infected peripheral blood lymphocytes
- 20 Exposed gp120 antigens
- 22 Antibodies non-competitively bound to the gp120 antigen on the HIV infected blood lymphocytes
- 24 Uninfected peripheral blood lymphocytes
- 26 Magnetic gradient
- 28 Focused light source.

## PREFERRED EMBODIMENT—DESCRIPTION OF FIGS. 1 THROUGH 4

FIG. 1 shows the sample of several drops of whole blood diluted in approximately 0.5 cc PBS 10. To the diluted

sample is added the Murine anti-gp120 monoclonal antibodies coupled to paramagnetic microspheres 14, and the Fluorescein conjugated anti-gp120 polyclonal antibodies IgG 16. In the sample of diluted whole blood are a small number of HIV-infected peripheral blood lymphocytes 18, bearing CD4, and also bearing numerous exposed gp120 antigens 20 (for simplicity, the Figures only display one gp120 antigen per peripheral blood lymphocyte).

FIG. 2 shows the mixture of blood and antibodies after incubation for 5 minutes; both antibodies non-competitively bound to the gp120 antigen on the HIV infected blood lymphocytes 22. This renders each HIV infected peripheral blood lymphocyte 18 coated with both the Murine anti-gp120 monoclonal antibodies coupled to paramagnetic microspheres 14 and the Fluorescein conjugated anti-gp120 polyclonal antibodies IgG 16. The uninfected peripheral blood lymphocytes 24 remain uncoated by either of the antibodies.

FIG. 3 shows the vessel containing the mixture of incubated whole blood diluted in approximately 0.5 cc phosphate buffered saline (PBS) 10 and reagents exposed to a strong magnetic gradient 26 at a predetermined point on the outer surface of the reaction vessel. The magnetic field causes the migration of antibodies non-competitively bound to the gp120 antigen on the HIV infected blood lymphocytes 22 to the inner surface of the reaction vessel at the maximum point of concentration of the magnetic gradient 26, thus separating the HIV-infected peripheral blood lymphocytes 18 from the uninfected peripheral blood lymphocytes 24 in the diluted whole blood sample. The magnetic separation takes approximately 20 seconds.

FIG. 4 shows the vessel after the designated time for magnetic separation to occur. The predetermined point of maximum magnetic concentration at the gradient 26 is illuminated by a suitable focused light source 28 at 488 nm wavelength, causing all antibodies non-competitively bound to and to the gp120 antigen on the HIV infected blood lymphocytes 22 to glow and to separate from the uninfected peripheral blood lymphocytes 24 in the whole blood sample 10. Fluorescein conjugated anti-gp120 polyclonal antibodies IgG 16 provide a low intensity diffuse background fluorescence.

#### Reagents

1. Immunodiagnostics, Inc., Product #1121-M: Murine Anti-gp120 HIV-1 mAb Coupled to Paramagnetic Microspheres.

#### Previous Uses:

- B and T cell research
- Ultrastructural analysis
- B and T cell panning
- Immunoprecipitation

These monoclonal antibodies of mouse origin are highly specific with high affinity to the gp120 HIV-1 glycoprotein. They are cross-reactive and cross-neutralizing antibodies, which are covalently bonded to paramagnetic microspheres. Their coupling ratio is approximately 2.5 micrograms of protein per milligram of magnetic microspheres. Specificity testing demonstrates that the Magnetic Murine anti-gp120 mAb binds recombinant gp120 (MN, IIIB) peroxidase conjugate as determined by ELISA. The biological activity is defined as the binding of these antibodies to CD-4 bearing, HIV-1 infected cells and HIV-1 infected human peripheral blood lymphocytes.

2. Immunodiagnostics, Inc. Product #1301-F: Fluorescein Rabbit Anti-gp120 HIV-1 IIIB pAb IgG.

#### Previous Uses:

- Dot Blot assays
- FACS
- Immunohistocytology
- Direct immunofluorescence assays

These Fluorescein conjugated anti-gp120 (HIV-1 IIIB) pAb IgG were highly purified (95% pure) polyclonal IgG before use for FITC conjugation. The conjugate was then further purified by gel exclusion chromatography. The specificity of this fluorescein conjugated pAb IgG is defined by its binding to native and recombinant HIV-1 gp120 in Dot Blot assays and by its staining of cell surfaces in direct immunofluorescence assays. This reagent may be used for direct immunofluorescence assays. This reagent may be used for direct immuno-fluorescent staining of cells in the 1:50 dilution range, while Dot Blot assays with purified gp 120 may be performed at a minimum dilution of 1:100.

Both monoclonal and polyclonal antibodies bind to the V3 loop of the HIV-1 envelope glycoprotein gp120. The are not competitive, which means they attach to different regions of the V3 loop of gp120. This factor permits them to be used simultaneously for their specific and different purposes.

#### Preferred Embodiment—Operation

A sample of whole blood diluted in approximately 0.5 cc phosphate buffered saline 10 is combined with the reagents in a vessel. The first reagent consists of murine anti-gp120 monoclonal antibodies coupled to paramagnetic microspheres 14. The second reagent consists of fluorescein conjugated anti-gp120 polyclonal antibodies IgG 16. The reagents are designed to bind themselves to HIV infected blood lymphocytes 18, bearing CD-4, which has numerous exposed gp120 antigens 20, which act as the connection points.

The mixture of blood and reagents is incubated at 37 degrees centigrade for approximately 5 minutes. After incubation the antibodies non-competitively bind to the gp120 antigen on the HIV infected blood lymphocytes 22. This renders each HIV infected peripheral blood lymphocyte 18 coated with both murine anti-gp120 monoclonal antibodies coupled to paramagnetic microspheres 14 and the fluorescein conjugated anti-gp120 polyclonal antibodies IgG 16. The uninfected peripheral blood lymphocytes 24 remain uncoated by either of the antibodies.

A strong magnetic gradient 26 is applied to a predetermined point on the outer surface of the vessel. The magnetic field causes migration of all antibodies non-competitively bound to the gp120 antigen on the HIV infected blood lymphocytes 22 to the inner surface of the vessel at the maximum point of concentration of the magnetic gradient 26, thus separating them from the uninfected peripheral blood lymphocytes 24 in the whole blood sample 10. The magnetic separation takes approximately 20 seconds.

After separation of antibodies non-competitively bound to the gp120 antigen on the HIV infected blood lymphocytes 22 from the uninfected peripheral blood lymphocytes 24, the predetermined point of maximum concentration of the magnetic gradient 26 is illuminated by a suitable focused light source 28 at 488 nm wavelength, causing all of the antibodies non-competitively bound to the gp120 antigen on the HIV infected blood lymphocytes 22 now aggregated at the predetermined point to glow at between 530–540 nm in fluorescent light.

Likewise, the excess of magnetic particles unbound immunologically to cell surfaces will travel at a much greater velocity to the inner surface of the vessel wall, assuring that before any cell coated with magnetic particles

arrives at the vessel wall, there will have formed a dark coating of unbound Murine anti-gp120 monoclonal antibodies coupled to paramagnetic microspheres 14, against which the infected cells will adhere, also providing a nice contrast for the high density of glowing antibodies non-competitively bound to the gp120 antigen on the HIV infected blood lymphocytes 22. Polyclonal antibodies IgG 16 unbound to HIV infected peripheral blood lymphocytes 18 in the sample of diluted blood, are of a volume only sufficient to provide only a low intensity diffuse background fluorescence as compared to the high intensity of the antibodies non-competitively bound to the gp120 antigen on the HIV infected blood lymphocytes 22 visible by fluorescence microscopy on the infected cells adhering to the inner surface of the reaction vessel wall.

#### Conclusions, Ramifications, and Scope

Accordingly, it can be seen that we have developed a fluorometric immunological assay method for HIV-1 infection using two reagents: one coupled with paramagnetic microspheres and the other with an FITC to separate and identify the HIV-infected peripheral blood lymphocytes 18 in diluted whole blood 10 by separating the cells using a magnetic gradient 26 and causing them to fluoresce using a focused light source 28.

The advantages of the invention are:

- a. Cell-bound antigen-based test closes the window period created by having to rely on the host immune system to produce antibodies against HIV-1 antigens to around 4 days.
- b. Appearance of cell-bound gp120 parallels appearance of viral genetic material, enabling invention to detect HIV presence in same time period as the PCR test at a small fraction of the cost.
- c. Entire test procedure requires minutes to turn around compared with weeks for PCR test.
- d. Increased accuracy and low cost allow it to act as both screening and confirmatory test.
- e. Can also be utilized in an automated format, utilizing a multi-purpose cartridge and fully automated incubator, magnetic separator and imaging system, permitting operation by non-medically trained personnel.
- f. Test can be contained in a blood collection cartridge to permit complete, self-contained, disposable unit that is much easier to handle than the PCR test for viral genetic material.

Although the description above contains many specificities, these should not be construed as limiting the scope of the invention but as merely providing illustrations of some of the presently preferred embodiments of this invention. Various other embodiments and ramifications are possible within its scope. For example, the method can be used to test for other viral infections by varying the antibody combinations, or other fluorochromes could be utilized. The method can also be used test to for water contamination, or to separate and identify cancer cells.

Thus, the scope of the invention should be determined by the appended claims and their legal equivalents, rather than by the examples given.

We claim:

1. A method of detecting an HIV-infected cell in an aqueous sample comprising the steps of,
  - a) combining a first anti-gp120 antibody attached to a magnetic particle; a second anti-gp120 antibody attached to a detectable label; and an aqueous sample containing HIV-infected peripheral blood lymphocytes displaying gp120 on the cell surface, to form a mixture;
  - b) incubating said mixture under conditions effective for binding of said antibodies to said gp120 to form a

complex, said complex comprising said first and second antibody bound to a HIV-infected cell on said magnetic particle; and

- c) moving said magnetic particle to a predetermined point on a reaction vessel holding said mixture, wherein said moving is accomplished by a magnetic field acting on said magnetic particle;
- d) detecting the label of said second antibody bound to gp120 on said HIV-infected cell, with the proviso that no step of washing of said mixture and no step of removing unbound first antibody and unbound second antibody from said mixture is performed in steps a), b), c), and d).
2. A method of claim 1, wherein said first and second antibody recognize different regions of gp120.
3. A method of claim 1, wherein said aqueous sample is whole blood.
4. A method of claim 1, wherein said predetermined point is illuminated with a light effective to detect said label.
5. A method of claim 1, wherein said detectable label is FITC.
6. A method of claim 1, wherein said first antibody is a monoclonal antibody.
7. A method of claim 1, wherein said second antibody is a polyclonal antibody.
8. A method of claim 1, where said first antibody is a monoclonal antibody and said second antibody is a polyclonal antibody coupled to a detectable label which is FITC.
9. A method of detecting an HIV-infected cell in an aqueous sample comprising the steps of,
  - a) combining a first anti-gp120 antibody attached to a magnetic particle; a second anti-gp120 antibody attached to a detectable label; and an aqueous sample containing HIV-infected cells displaying gp120 on the cell surface, to form a mixture;
  - b) incubating said mixture under conditions effective for binding of said antibodies to said gp120 to form a complex, said complex comprising said first and second antibody bound to a HIV-infected cell on said magnetic particle; and
  - c) moving said magnetic particle to a predetermined point on a reaction vessel holding said mixture, wherein said moving is accomplished by a magnetic field acting on said magnetic particle;
  - d) detecting the label of said second antibody bound to gp120 on said HIV-infected cell, with the proviso that no step of washing of said mixture and no step of removing unbound first antibody and second antibody from said mixture is performed in a), b), c), and d).
10. A method of claim 1, wherein said HIV-infected cell is a peripheral blood lymphocyte.
11. A method of claim 9, wherein said first and second antibody recognize different regions of gp120.
12. A method of claim 9, wherein said aqueous sample is whole blood.
13. A method of claim 9, wherein said predetermined point is illuminated with a light effective to detect said label.
14. A method of claim 9, wherein said detectable label is FITC.
15. A method of claim 9, wherein said first antibody is a monoclonal antibody.
16. A method of claim 9, wherein said second antibody is a polyclonal antibody.
17. A method of claim 9, where said first antibody is a monoclonal antibody and said second antibody is a polyclonal antibody coupled to a detectable label which is FITC.

\* \* \* \* \*



US 20010008760A1

(19) **United States**(12) **Patent Application Publication** (10) **Pub. No.: US 2001/0008760 A1**  
**KING et al.** (43) **Pub. Date: Jul. 19, 2001**(54) **REAGENT SYSTEM AND KIT FOR  
DETECTING HIV INFECTED CELLS**is a continuation-in-part of application No. 08/732,  
784, filed on Oct. 15, 1996, now Pat. No. 5,714,390.(76) **Inventors: CHESTER F. KING, FREDERICK,  
MD (US); ROBERT A.  
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ARLINGTON, VA 22201 (US)**(57) **ABSTRACT**(\*) **Notice:** This is a publication of a continued pro-  
secution application (CPA) filed under 37  
CFR 1.53(d).(21) **Appl. No.: 09/139,663**(22) **PCT Filed: Oct. 15, 1997**(86) **PCT No.: PCT/US97/18649****Related U.S. Application Data**(63) **Continuation-in-part of application No. 08/732,782,  
filed on Oct. 15, 1996, now Pat. No. 5,817,458, which**

This invention relates to blood collection and diagnostics. More particularly, the invention relates to blood collection and diagnostics utilizing techniques such as magnetic separation and photodetection. The present invention also relates to methods and an apparatus for detecting the presence of antigens displayed on the surface of cells. More preferably, the present invention relates to the detection of cells infected by human immunodeficiency virus (HIV) and related viruses. In accordance with the present invention, HIV-infected cells can be detected and separated from uninfected cells. In a preferred embodiment, separation is achieved by a magnetic field. By coating the infected cells with magnetic particles, transfer of the cells to a precise location is facilitated. A novel aspect of the present invention is a cartridge antigen test which allows for the collection and mixing of blood with reagents in one package, which can be viewed on a fluorescent microscope.



FIG. 1

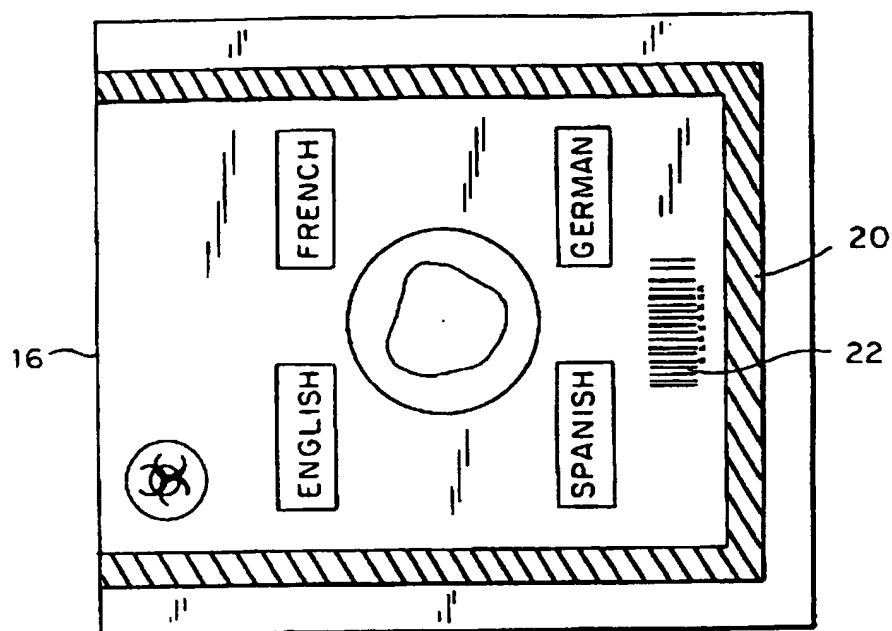


FIG. 2

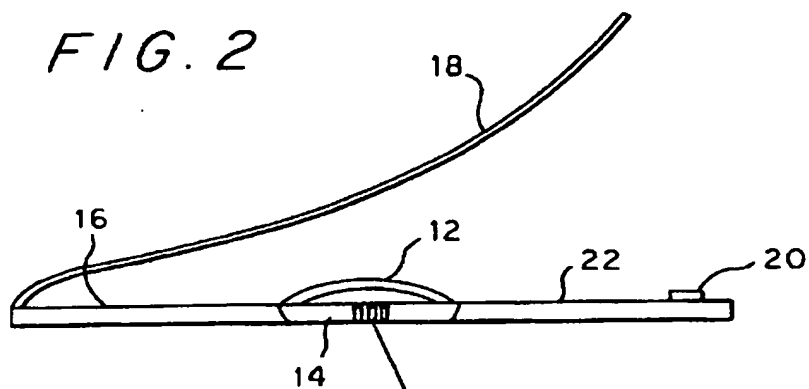
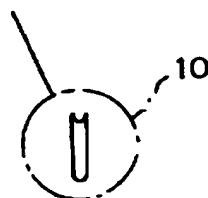


FIG. 3



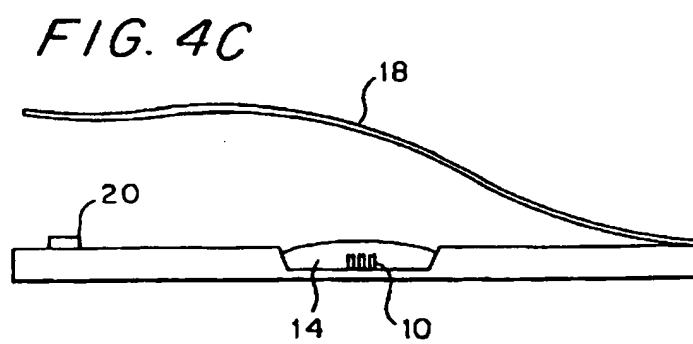
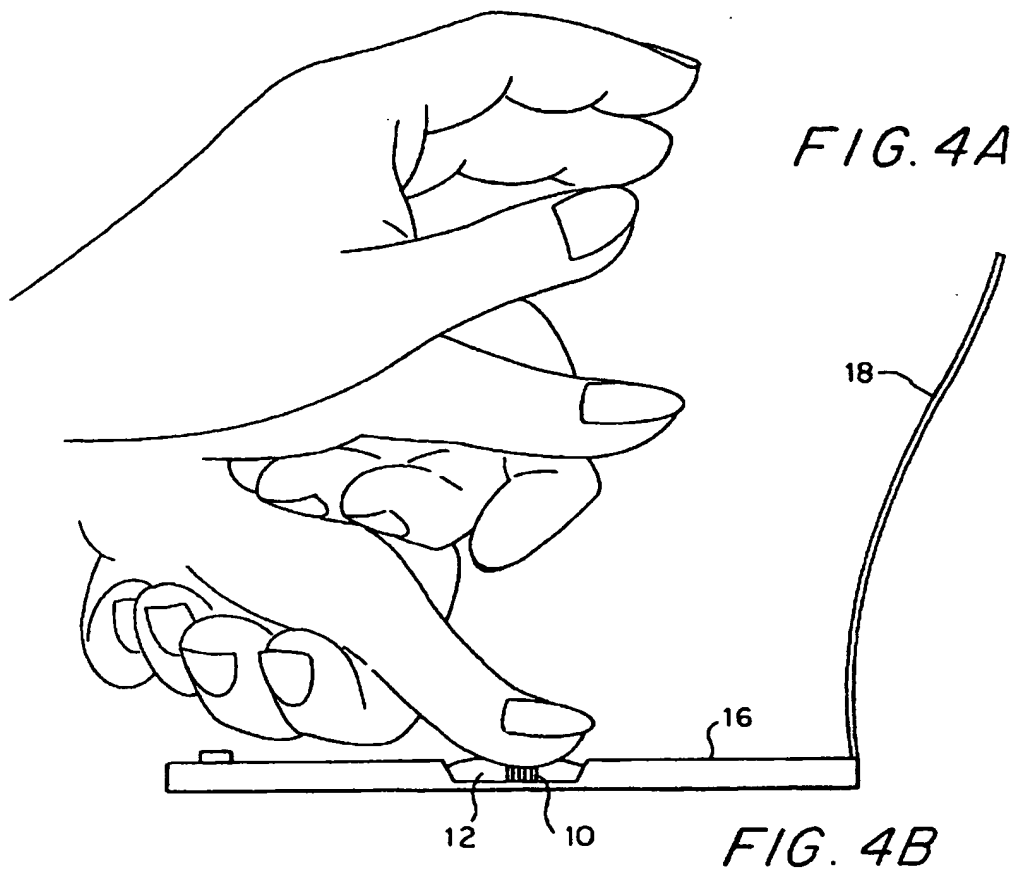


FIG. 5A

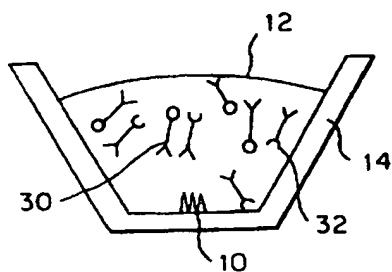


FIG. 5C

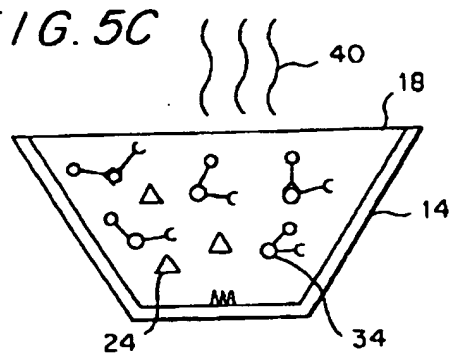


FIG. 5B

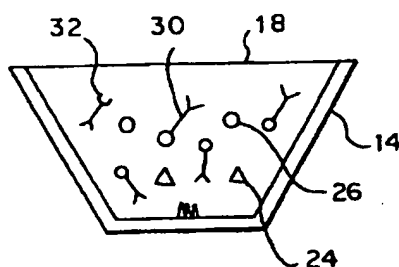


FIG. 5D

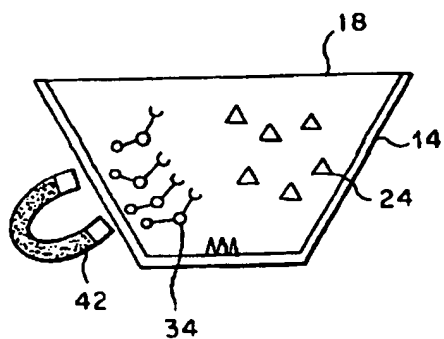


FIG. 5E

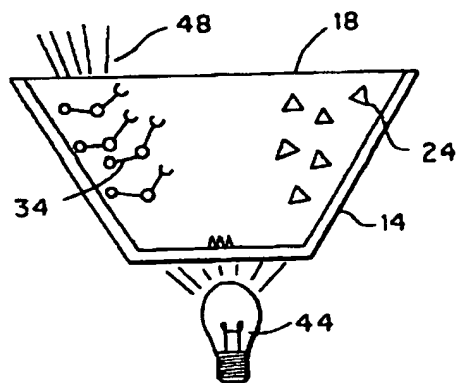


FIG. 6A

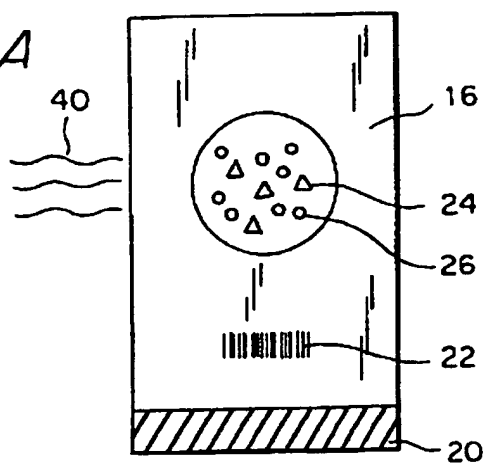


FIG. 6B

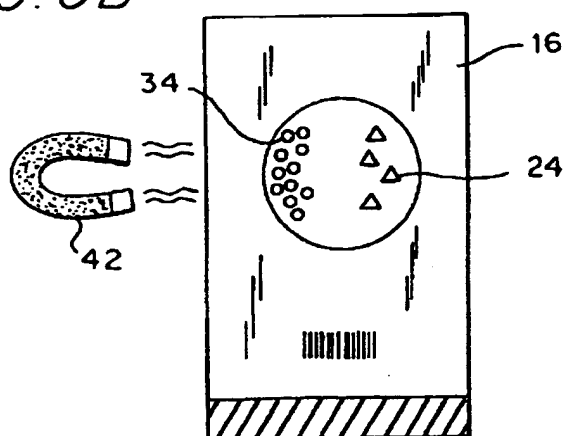
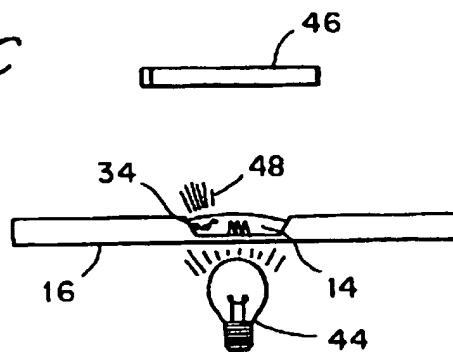


FIG. 6C



## REAGENT SYSTEM AND KIT FOR DETECTING HIV INFECTED CELLS

### CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application is a continuation-in-part of PCT/US97/18649 filed Oct. 15, 1997, which is a continuation in part of U.S. application No. 08/732,782 filed Oct. 15, 1996, and U.S. application No. 08/732,784 filed Oct. 15, 1996.

### FIELD OF INVENTION

[0002] The present invention relates to methods for detecting the presence of antigens displayed on the surface and the interior compartments of cells. More preferably, the present invention relates to the detection of cells infected by micro-organisms, viruses, etc., such as human immunodeficiency virus (HIV), other retroviruses, DNA viruses, RNA viruses, and non sexually-transmitted viruses. Detection of infection is achieved earlier and more accurately than with previous methods. In accordance with the present invention, virus infected cells can be detected and separated from uninfected cells. In a preferred embodiment, separation is achieved by a magnetic field. By coating the infected cells with magnetic particles, transfer of the cells to a precise location is facilitated.

[0003] The present invention relates to methods and materials useful wherever the isolation and quantitation of infected cells is of value, such early diagnosis of HIV infection, determination of the infectivity of a specific strain or isolate of HIV-1 on a specific target cell population in tissue culture, the assessment of the ability of neutralizing antibodies or sera or drugs to decrease infectivity of said viral strain, etc. More particularly, in one embodiment, the invention provides compositions and methods for utilizing a cocktail or monoclonal or polyclonal anti-surface protein antibodies conjugated with a tag, such as FITC, and beads coated with an antibody produced against the tag, such as anti-FITC microbeads.

### BACKGROUND

[0004] The state of the art with respect to the epidemiology and immunology of the causative agent of AIDS in humans is well summarized in: Laurence, "The Immune System and AIDS," *Scientific American*, December, 1985, pp. 84-93; Gallo, "The First Human Retrovirus," *Scientific American*, December, 1986, pp. 88-98; Gallo, "The AIDS Virus," *Scientific American*, January, 1987, pp. 47-56; Levy et al., *Science*, 225, 840-842 (1984); "Mobilizing against AIDS," Institute of Medicine, National Academy of Sciences, Harvard University Press (Cambridge Mass. 1986); and Lane et al., *Ann Rev. Immunol.*, 3, pp.477-500 (1985).

[0005] The role of the CD4 surface glycoprotein of human T lymphocytes in infection by HIV has been extensively studied as represented by Dalgleish et al., *Science*, 312, pp. 763-767 (1984); Klatzmann et al., *Science*, 267-268 (1984); Klatzmann et al., *Science*, 225, pp. 59-62 (1984); McDoual et al., *J. Immunol.*, 135, pp.3151-3162 (1985); and Maddon et al., *Cell*, 47, pp.333-348 (1986).

[0006] Infection of a T-cell or macrophage or monocyte (CD4 bearing mono-nuclear cells) follows from interaction between an epitope borne by HIV-1 and the CD4 receptor

which is located ultimately on the cell surface. The epitope on HIV-1 is borne by the envelope glycoprotein gp120 (molecular weight 120 kilodaltons). The glycoprotein GP 120 is structurally exposed on the outside of the HIV-1 envelope. The gp 120/gp41 binds and fuses to the target cell, permitting the injection of the viral genome into the target cell. After a complex process of decoding each strand of RNA and creating complementary DNA, which is then integrated into the host cell genome, the cell begins to produce all of the components of new viruses. The production of the envelope proteins of the viruses begins inside the cell and rapidly proceeds to the expression of gp120/41 complex intermingled with host cell membrane after leaving the endoplasmic reticulum on the target cell surface.

[0007] In light of the above background information regarding HIV and AIDS, it can be deduced that antibodies specific for the envelope of the virus, which plays such an important role in the establishment of the viral infection, could have great significance in identifying the most crucial cell-bound antigens on the surface of infected cells in the peripheral blood.

[0008] A number of research groups have reported successful development of murine monoclonal antibody specific for gp120. For example, T. C. Can et al. (*Eur. J. Immunol.* 16:1465, 1986) reported that they chemically synthesized a portion of the peptide chain of gp120 and then prepared monoclonal antibodies (MAbs) specific for the synthetic peptide. They employed those MAbs in an indirect fluorescent antibody technique and reported they were able to detect HIV infection with greater sensitivity than was possible with the reverse transcriptase determination technique. Additional reports of murine anti-gp 120 MAbs have been reported by Gostling et al., (*J. Clin. Microbiol.*: 25,845, 1987) and Matsushida et al., (*Medical Immunol.* 14: 307, 1987).

[0009] Testing serum for antibodies to HIV is currently the most cost-effective and accurate method of screening for and confirmation of infection. References 1-5 "AID Knowledge Base 2.1-9, Centers for Disease Control—Update: Serologic Testing for Antibody to Human Immunodeficiency Virus", *MMWR* 1988; 36: 833-40; Schwartz, J. S., Dans, P. E., "Kinosian BP Human Immunodeficiency Virus Test Evaluation, Performance and Use", *JAMA* 1988; 259: 2574-9; Burke, D. S., Brundage, J. F., Redfield, R. R. et al., "Measurement of the False Positive Rate in a Screening Program for Human Immunodeficiency Virus Infections", *New England Journal of Medicine*, 1988; 319: 961-4; Cohen, N. D., Munoz, A., Reitz, B. A. et al. "Transmission of Retroviruses by Transfusion of Screened Blood in Patients Undergoing Cardiac Surgery", *New England Journal of Medicine* 1989; 320: 1172-6; MacDonald, K. L., Jackson, J. B., Bowman, R. J. et al., "Performance Characteristics of Serologic Tests for Human Immunodeficiency Virus Type 1 (HIV-1) Antibody Among Minnesota Blood Donors. Public Health and Clinical Implications", *Ann Intern Med.* 1989; 110: 617-21.

[0010] HIV antibody tests have their limitations. Usually antibodies to HIV appear within 3-6 months and as early as 6-8 weeks, but silent infections have been documented in which seroconversion has occurred as late as 3 years from the moment of exposure. Therefore, because an infected person does not develop antibodies immediately, a negative test result cannot rule out HIV infection.

[0011] It has been shown that the majority (90%) of people first testing positive for HIV will develop AIDS within one year. This strongly suggests that the average person identifying HIV infection has been positive for an average of 8-9 years, in view of the fact that the average interval between infection and AIDS is 9-10 years.

[0012] This is particularly problematic because of the behavioral studies indicating that a person practicing high risk behaviors is likely to seek testing within days or a few weeks of the high risk behavior. That person is then likely to forget about HIV and continue risky behavior based on the false reassurance of a negative test performed before seroconversion was even possible.

[0013] The consequences of the above observations are: 1. The majority of people practicing intermittent high risk behaviors tend to seek reassurances very shortly after committing such behaviors; 2. They get reassurance and false security because of false-negative tests based on as yet undetectable antibody levels; 3. The majority of infected people continue intermittent or continuous high risk behaviors for 8-9 years after becoming infected; 4. These people are, therefore, transmitting HIV for 8-9 years; 5. If affordable accurate testing could be accomplished within the brief interval between risky behavior and seroconversion, a significant increase in early HIV detection would be likely; 6. Therefore, affordable early detection would create a significant reduction in the high prevalence of HIV transmission by the falsely assured and oblivious people in the 90% majority cited above.

[0014] In a study of consistent sequential detection of RNA, antigen and antibody in early HIV infection, sequential appearance in blood of HIV RNA, HIVAg, and HIV antibody was found. Data derived from testing Seroconversion Panels demonstrate a consistent sequential rise in the concentrations of HIV RNA followed by HIV antigen (p24), followed by anti-HIV in early HIV infection. Based on the timing of the appearance of RNA and antigen it was concluded that HIV RNA and HIVAg could be used to confirm early infection. RNA and/or HIVAg tests were concluded to be potentially useful for earlier detection of HIV infection (e.g., blood screening). Busch, M., Schumacher, Richard T., Stramer, S., et. al. "Consistent Sequential Detection of RNA, Antigen and Antibody in Early HIV Infection: Assessment of the Window Period" Irwin Memorial Blood Center, San Francisco, Calif., Boston Biomedical, Inc., Bridgewater, Mass., Poster presented at XI International AIDS Conference, Vancouver, BC July 1986.

[0015] Efforts have been made to close this "window" between exposure and antibody detect ability. The p24 antigen test has already been mandated for use by all registered blood and plasma centers because of a partial closure of the "window" achieved by this method. However, in the best-case scenario, p24 antigen detection realistically only closes the window by 6-7 days. A syndrome of primary infection has been shown to occur in the majority of HIV-1 infected patients between days 5 and 30 following exposure to and infection with the HIV-1 virus. During this interval a very intense viremia has been discovered, and as well, an outpouring of peripheral blood mononuclear cells occurs (Dr. Mark Lewis, personal communication), many of which are suggested to be productively infected. In addition animal studies using the SHIV (HIV-1 envelope with SIV core)

model demonstrate that following the vaginal inoculation, SHIV is detectable in the blood stream with sensitive culture techniques within 2 days of exposure (Dr. Mark Lewis, personal communication). Since a significant rate of viral reproduction occurs within the first week of infection causing the presence of the envelope glycoprotein GP 120 bearing lymphocytes in the peripheral blood, detection of blood-bound GP 120 is an effective means to close the "window" even further than the p24 test.

[0016] A method for determining the concentration of substances in biological fluids (e.g., drugs, hormones, vitamins and enzymes) wherein magnetically responsive, permeable, solid, water insoluble, micro particles are employed is disclosed in U.S. Pat. No. 4,115,534. Functional magnetic particles formed by dissolving a mucopolysaccharide such as chitosan in acidified aqueous solution containing a mixture of ferrous chloride and ferric chloride is disclosed in U.S. Pat. No. 4,285,819. The micro spheres may be employed to remove dissolved ions from waste aqueous streams by formation of chelates. U.S. Pat. No. 3,933,997 describes a solid phase radio immunoassay for digoxin where anti-digoxin antibodies are coupled to magnetically responsive particles.

[0017] Small magnetic particles coated with an antibody layer are used in U.S. Pat. No. 3,970,518 to provide large and widely distributed surface area for sorting out and separating select organisms and cells from populations thereof. U.S. Pat. No. 4,018,886 discloses small magnetic particles used to provide large and widely distributed surface area for separating a select protein from a solution to enable detection thereof. The particles are coated with a protein that will interact specifically with the select protein.

[0018] U.S. Pat. No. 4,070,246 describes compositions comprising stable, water insoluble coatings on substrates to which biologically active proteins can be covalently coupled so that the resulting product has the biological properties of the protein and the mechanical properties of the substrate, for example, magnetic properties of a metal support.

[0019] A diagnostic method employing a mixture of normally separable protein-coated particles is discussed in U.S. Pat. No. 4,115,535. Micro spheres of acrolein homopolymers and copolymer with hydrophilic comonomers such as methacrylic acid and/or hydroxyethylmethacrylate are discussed in U.S. Pat. No. 4,413,070. U.S. Pat. No. 4,452,774 discloses magnetic iron-dextran micro spheres which can be covalently bonded to antibodies, enzymes and other biological molecules and used to label and separate cells and other biological particles and molecules by means of a magnetic field. Coated magnetizable micro particles, reversible suspensions thereof, and processes relating thereto are disclosed in U.S. Pat. No. 4,454,234. A method of separating cationic from anionic beads in mixed resin beds employing a ferromagnetic material intricately incorporated with each of the ionic beads is described in U.S. Pat. No. 4,523,996. A magnetic separation method utilizing a colloid of magnetic particles is discussed in U.S. Pat. No. 4,526,681. U.K. Patent Application GB No. 2,152,664A discloses magnetic assay reagents.

[0020] An electron-dense antibody conjugate made by the covalent bonding of an iron-dextran particle to an antibody molecule is reported by Dutton et al. (1979) *Proc. Natl. Acad. Sci.* 76:3392-3396. Ithakissios et al. describes the use

of protein containing magnetic micro particles in radioassays in *Clin. Chem.* 23:2072-2079 (1977). The separation of cells labeled with immunospecific iron dextran micro spheres using high gradient magnetic chromatography is disclosed by Molday et al. (1984) *FEBS*, 17: 232-238. In *J. Immunol. Meth.* 52:353-367 (1982) Molday et al. describe an immunospecific ferro-magnetic iron-dextran reagent for the labeling and magnetic separation of cells. An application of magnetic micro spheres in labeling and separation of cells is also disclosed by Molday et al. in *Nature* 268:437-437 (1977). A solid phase fluoroimmunoassay of human albumin and biological fluids is discussed by Margessi et al. (1978) *Clin. Chim. Acta.* 89:455-460. Nye et al. (1976) *Clin. Chim. Acta.* 69:387-396 discloses a solid phase magnetic particle radioimmunoassay. Magnetic fluids are described by Rosenweig (1983) *Scien. Amer.* 10:136-194. Magnetic protein A micro spheres and their use in a method for cell separation are disclosed by Widder, et al. (1979) *Clin. Immunol. and Immunopath.* 14:395-400.

[0021] U.S. Pat. No. 5,279,936 is a method directed to the separation of a component of interest from other components of a mixture by causing the binding of the component of interest to magnetic particles. In the embodiment of the invention which is a method to separate cells from a mixture containing other components, the method comprises layering a first liquid medium containing cells and other components with a second medium which is of a different density than and/or different viscosity than the first liquid medium. The cells are bound to paramagnetic particles. The layered first liquid medium and the second liquid medium are subjected to a magnetic field gradient to cause the cell particles to migrate into the second medium. The purpose of isolating the cells in the second liquid medium is to then by a further embodiment to separate the cells from the second liquid medium. In the current invention, there is no need for a second liquid medium because the magnetic separation of HIV-1 infected cells is accomplished in the medium of PBS diluted blood, by bringing the infected cells to a predetermined point in the reaction vessel. The only task required after separation is the illumination of the point of highest magnetic field concentration, to ascertain the presence or absence of high density specific fluorescence, which if present would indicate the presence of fluorescently tagged HIV infected peripheral blood leucocytes (pb1).

[0022] U.S. Pat. No. 4,935,147 is a method that specifically targets the application of magnetic separation in the assay of organic and inorganic biochemical analytes, particularly those analytes of interest in the analysis of body fluids. The method of the mentioned patent provides a way of separating non-magnetic particles from a medium by virtue of the chemically controlled non-specific reversible binding of such particles to magnetic particles. Because of the small size of the magnetic particles, it also provides for a very rapid binding of a substance to be separated. By then aggregating the particles there is provided a much more rapid and complete magnetic separation than has been achieved by previous methods. In the current invention, this technique of magnetic separation does not apply because of the fact the antigen of interest is bound to cells, and therefore not in solution or in need of agglutination for separation. The current invention merely requires the adherence of the many magnetic particles to an infected cell surface to magnetically pull the entire cell of interest to a predetermined point in the reaction vessel for viewing.

[0023] With respect to a kit, the prior art collected blood for testing in multiple steps. The first step was to collect the blood into a suitable container from a puncture wound in the skin of a finger or by venipuncture. Then the blood would have to be placed into a container suitable for transporting or mixing with test reagents. Then reagents would have to be added in a multiple step fashion, interrupted by wash steps. The problem with this approach is multiple steps which are time consuming and require training. In the collection of blood, the prior art is still dealing with the lance and test tube methods.

[0024] For example, the aforementioned U.S. Pat. 4,777, 964 to David Briggs, Kent A. Leger, Brenda Briggs (Oct. 18, 1988) provides a system for whomever wishes to ascertain whether or not he is carrying the AIDS virus to perform a blood sampling and to forward the sample to a lab for the further testing. The kit contains a lance and a tube for collecting the sample and requires the user to seal the tube at the ends with putty. This device and kit is only a means for collecting blood and keeping the sample intact for mailing to a laboratory for further testing. No tests are performed using the appliances provided. In addition, the sample must be transferred to a testing vessel and mixed with the appropriate testing medium. There are a host of other test kits and methods for collection and preparation of specimens. The following patents are of interest with respect to this field: U.S. Pat. No. 4,382,062 to Kohl (May 3, 1983); U.S. Pat. No. 4,365,970 to Lawrence et al. (Dec. 28, 1982); U.S. Pat. No. 4,122,947 to Falla Oct. 31, 1978); U.S. Pat. No. 3,272,319 to Brewer (Sep. 13, 1966); U.S. Pat. No. 3,203,540 to Kalt et. al. (Aug. 31, 1965). None of the above-mentioned patents provide sample collection, preparation and observation of the immunochemical reaction in the same vessel.

[0025] Some test media provide for the performance of the magnetic separation, but do not provide for the reaction to occur in the collection apparatus, nor can the complete test be performed outside a controlled laboratory environment where multiple steps must be performed. U.S. Pat. No. 5,186,827 to Paul A. Liberti, Brian P. Feeley, Dhanesh I. Gobel (Feb. 16, 1993) describes an elaborate magnetic separator for separating magnetic particles from a non-magnetic test medium. The magnetic separator includes a non-magnetic container having a peripheral wall with an internal surface area for receiving the test medium, and magnetic means for generating a magnetic field gradient within the container in which tested material is contained in reaction vessels such as test tubes.

[0026] There are also methods that utilize magnetic separation and the use of light sources to identify particles. U.S. Pat. No. 5,238,810 to Koichi Fujiwara, Juichi Noda, Hiroko Misutani, Hiromichi Mizutani (Aug. 23, 1993) provides for such a process; however, as with other magnetic separation methods, this method involves multiple apparatus and steps just to collect and prepare the blood samples for testing. This method also focuses on using one reagent for its test, rather than on a double reagent mixture. It provides for various vessel configurations for performing the reaction, but does not include or contemplate a vessel that has served as reagent storage, blood collection, mixture, incubation and viewing device in one.

## SUMMARY OF THE INVENTION

[0027] The present invention relates to a variety of assays for detecting and/or separating, e.g., pbbs, T-cells, immortalized cell lines, macrophages, monocytes, CD4 bearing cells, artificial liposomes, etc., utilizing magnetic separation technology. In a particular aspect, it relates to obtaining a blood sample and mixing it with testing reagents in one step, and in one disposable vessel. The vessel can be incubated and the related results of reaction between the reagents and the blood sample can be viewed and read in the vessel by a fluorescent microscope without additional processing for quick and accurate testing.

[0028] The present invention is directed to blood collection and magnetic separation apparatus and methods in which antibody-coupled magnetic particles and antibody-conjugated fluorochromes are used to isolate substances of interest from a non-magnetic test medium by means of high gradient magnetic separation and identification by application of focused light.

[0029] An aspect of the present invention relies upon a unique reaction vessel that serves the multiplicity of purposes as stated above. The prior methods of magnetic separation differ in various ways, e.g., because of the incompatibility of reaction vessel configuration with blood sample collection and single-step testing. In addition, most magnetic separation devices do not provide for viewing any further reaction within the vessel.

[0030] The current invention provides a self-contained micro-baggy of reagents that is punctured and permitted to mix with the sample of blood at the same time the sample is being collected. Further, the chamber in which the blood is collected, and in which the reagents are mixed with the blood, is also the same chamber or vessel used for incubating the reaction mixture, and further, is the chamber in which magnetic separation of the infected cells, if present, is performed. Finally it also serves as the chamber in which the infected cells, if present, are viewed. There is no equivalent multipurpose chamber such as the present invention that provides for blood collection reagent storage, reagent/blood mixing, reaction incubation, magnetic separation and finally, viewing of any infected cells present.

[0031] The Cartridge Antigen Test (CAT) is a device that permits blood collection, reagent mixing with blood, incubation of the mixture, magnetic separation, and viewing of the test results. The device consists of a well slide with micro-lances, a micro-baggy full of reagents, a mylar cover strip, and a bar code for identification purposes.

[0032] The present invention also relates to a fluorometric immunoassay in which a pair of manufactured non-competitive antibodies to a surface antigen, such as gp120, are utilized. One antibody (Mab) is coupled to paramagnetic particles, while the second in conjugate with FITC. The present invention takes advantage of the technology of immunomagnetic separation developed over the past 15 years to enrich or separate out of a mixture of cells, specific cellular components based on their specific immunological markers. See, e.g., U.S. Pat. Nos. 4,777,145; 4,731,337; 5,186,827; 5,238,810; 5,279,936; 5,411,863; and 4,935,147.

[0033] In these inventions particular methods are disclosed for separating a substance from a liquid medium using magnetic particles. None of these inventions, however,

are specific for the process of using immunomagnetic particles for the diagnosis of HIV in whole blood. The present invention relies upon the commercial availability of high affinity anti-gp120 Mabs coupled with magnetic particles and a second non-competitive anti-gp120 Pab conjugated with FITC to fluorimetrically "tag" an HIV infected cell and then magnetically separate it from uninfected cells in whole blood.

[0034] Of particular importance to the background of the present invention is the consideration of factors that demonstrate the importance of creating a diagnostic system which takes advantage of the above described molecular biology of HIV infection. It is also important to understand the need for the present invention based upon the limitations posed by current screening and confirmatory test protocols which are still mainly dependent upon host immune response to HIV infection by antibody production.

[0035] Accordingly, several objects and advantages of our invention are the objective of placing the entire process of stabbing the finger, collecting the blood, treating the blood with test reagents, incubating the test mixture and reading the results form a single device with no transfers, additions, or complicated processes. The operator requires no special training to use the device. This allows for faster, automated testing of the results in remote sites and easy labeling of patient's tests and easy disposal of samples.

[0036] Still further objects and advantages will become apparent form a consideration of the ensuing description and accompanying drawings.

## BRIEF DESCRIPTION OF THE DRAWINGS

[0037] FIG. 1 is a frontal view of a collection/processing cartridge according to the present invention.

[0038] FIG. 2 is a side view of the collection/processing cartridge illustrating a well, microlances, a micro-baggy and a mylar cover.

[0039] FIG. 3 is an enlarged view of one of the microlances shown in FIG. 2.

[0040] FIG. 4A-4C are side views illustrating the collection of a blood sample from a test subject.

[0041] FIG. 5A-5E are side views of the well and illustrating an immunochemical reaction between blood and a two reagent system including incubation, application of a magnetic gradient, and the application of a focused light source on the reagent and blood mixture.

[0042] FIG. 6A-6C are top and side views respectively, of the cartridge and well illustrating incubation, the application of a magnetic gradient and a focused light source, and the observation of the reaction through a lens.

## DETAILED DESCRIPTION OF THE INVENTION

[0043] Several objects and advantages of our invention are to provide a cost-effective, accurate means of early (within 4 days of exposure) HIV-1 infection detection in whole blood that was based on the ability to immunochemically/magnetically isolate and fluorescently label HIV-1 infected peripheral blood lymphocytes. Advantages of the invention, include, e.g.: 1. cell-bound antigen-based test closes the



window period created by having to rely on the host immune system to produce antibodies against HIV-1 antigens to around four days; 2. Multi-purpose cartridge and fully automated incubator, magnetic separator and imaging system, permit operation by non-medically trained personnel; 3. Appearance of cell-bound gp120 parallels appearance of viral genetic material, enabling invention to detect HIV presence in same time period as "Gold Standard" PCR at a small fraction of the cost; 4. Functional design of the blood collection/ immunochemistry/ magnetic separation/ imaging cartridge permit complete, self contained, disposable unit that is much easier to handle than "gold standard" PCR test for viral genetic material; 5. Entire test procedure requires minutes to turn around compared with weeks for PCR; 6. Cost per test will be in tens of dollars rather than hundreds. Still further objects and advantages will become apparent from a consideration of the ensuing description and accompanying drawings.

[0044] In one aspect of the present invention (see, e.g., FIG. 5), a sample of several drops of whole blood is diluted with murine anti-gp120 monoclonal antibodies coupled to paramagnetic microspheres approximately 0.5 cc Phosphate Buffered Saline (PBS). To the diluted sample is added the Murine anti-gp120 monoclonal antibodies coupled to paramagnetic microspheres, and the Fluorescein conjugated anti-gp120 polyclonal antibodies IgG. In the sample of diluted whole blood are a small number of HIV infected peripheral blood lymphocytes, bearing CD-4, also bearing numerous exposed gp120 antigens. The mixture of blood and antibodies after incubation for five minutes, both antibodies are non-competitively bound to each and every gp 120 antigen. This renders each HIV infected peripheral blood lymphocytes coated with both the murine anti-gp120 monoclonal antibodies coupled to paramagnetic micro spheres and the Fluorescein conjugated anti-gp120 polyclonal antibodies IgG. The uninfected peripheral blood lymphocytes remain uncoated by either of the antibodies. A vessel containing the mixture of incubated blood and reagents can be exposed to a strong magnetic gradient at a predetermined point on the outer surface of the reaction vessel. The magnetic field causes the migration of all HIV infected peripheral blood lymphocytes to the inner surface of the reaction vessel at the maximum point of concentration of the magnetic gradient, thus separating the HIV infected peripheral blood lymphocytes from the uninfected peripheral blood lymphocytes in the diluted whole blood sample. The magnetic separation takes approximately 20 seconds. After the designated time for magnetic separation to occur, the predetermined point of maximum magnetic concentration is illuminated by a suitable focused light source at 488 nm wavelength, causing all HIV infected peripheral blood lymphocytes, now aggregated at the at a predetermined point to glow at between 520-540 nm fluorescent light. Although there will be an excess of Fluorescein conjugated anti-gp120 polyclonal antibodies IgG unbound to HIV infected peripheral blood lymphocytes in the sample of diluted blood, the volume is sufficient and dilution of Fluorescein conjugated anti-gp120 polyclonal antibodies IgG adequate to provide only a low intensity diffuse background fluorescence as compared to the high intensity of cell bound Fluorescein conjugated anti-gp120 polyclonal antibodies IgG visible by fluorescence microscopy on the infected cells adhering to the inner surface of the reaction vessel wall. Likewise, the excess of magnetic particles unbound immunologically to cell surfaces will

travel at a much greater velocity to the inner surface of the vessel wall, assuring that before any cell coated with magnetic particles arrive at the vessel wall, there will have formed a dark coating of unbound Murine anti-gp120 monoclonal antibodies coupled to paramagnetic micro spheres, against which the infected cells will adhere, also providing a nice contrast for the high density of glowing HIV infected peripheral blood lymphocytes.

[0045] Reagents can be obtained commercially, e.g., Immunodiagnostics, Inc., Murine Anti-gp120 HIV-1 mAb Coupled to Paramagnetic Microspheres. Monoclonal antibodies of mouse origin can be obtained commercially which are highly specific with high affinity to the gp120 HIV-1 glycoprotein. They are cross-reactive and cross neutralizing antibodies, which are covalently bonded to Paramagnetic Microspheres. Their coupling ratio is approximately 2.5 micrograms of protein per mg of magnetic microspheres. Specificity testing demonstrates that the Magnetic Murine anti-gp120 mAb binds recombinant gp120 (MN, IIIB) peroxidase conjugate as determined by ELISA. The biological activity is defined as the binding of these antibodies to CD-4 bearing, HIV-1 infected cells and HIV-1 infected human peripheral blood lymphocytes. Fluorescein Rabbit Anti-gp120 HIV-1 IIIB pAb IgG (e.g., Immunodiagnostics, Inc.) These Fluorescein conjugated anti-gp120 (HIV-1 IIIB) pAg IgG can be highly purified (95% pure) polyclonal IgG before use for FITC conjugation. The conjugate can then be further purified by gel exclusion chromatography. The specificity of this fluorescein conjugated pAb IgG can be defined by its binding to native and recombinant HIV-1 gp120 in Dot Blot assays and by its staining of cell surfaces in direct immunofluorescence assays. This reagent can be used for direct immunofluorescence assays. This reagent can be used for direct immuno fluorescent staining of cells in the 1:50 dilution range, while Dot blot assays with purified gp120 may be performed at a minimum dilution of 1:100.

[0046] Both monoclonal and polyclonal antibodies can be obtained (e.g., see above) which bind to the V3 loop of the HIV-1 envelope glycoprotein gp120 but which are not competitive, i.e., they attach to different regions of the V3 loop of gp120. This factor permits them to be used simultaneously for their specific and different purposes.

[0047] Further advantages of the above-described invention include, e.g., Cell-bound antigen-based test closes the window period created by having to rely on the host immune system to produce antibodies against HIV-1 antigens to around four days; Appearance of cell-bound gp 120 parallels appearance of viral genetic material, enabling invention to detect HIV presence in same time period as "gold standard" PCR at a small fraction of the cost; Entire test procedure requires minutes to turn around compared with weeks for PCR; Increased accuracy and low cost allow it to act as both screening and confirmatory test; This test can also be utilized in an automated format, utilizing a multi-purpose cartridge and fully automated incubator magnetic separator and imaging system, permitting operation by non-medically trained personnel; The test can be contained in a blood collection cartridge to permit complete, self contained, disposable unit that is much easier to handle than "gold standard" PCR test for viral genetic material.

[0048] Although the description above contains many specificities, these should not be construed as limiting the

scope of the invention but as merely providing illustrations of some of the presently preferred embodiments of this invention. Various other embodiments and ramifications are possible within its scope. For example, the method can be used to test for other viral infections by varying the antibodies combinations. In addition, other fluorochromes and detectable labels can be utilized. The method can be used to test for water contamination, the method can be used to separate and identify cancer cells.

[0049] Another aspect of the present invention relates to an assay for determining the presence of a desired antibody or other binding partner in a test sample. In a preferred embodiment, the desired antibody is an antibody generated against an antigen coded for by a virus, e.g., HIV-1, HIV-2, HTLV, HTLV EBV, CMV, SIV, etc. In this preferred embodiment, the antibody is a neutralizing antibody or an antibody able to interfere with infection of a cell by the virus. In this aspect of the invention, the presence of the antibody in the test sample is determined by its ability to interfere with infection of a target cell by a virus.

[0050] To perform this aspect of the invention, a target cell is contacted with a virus capable of infecting the cell. The step of contacting can be achieved, e.g., by combining the target cell and virus in a receptacle, such as a test tube, a slide well, a tissue culture flask, etc. Typically, the step of contacting is performed in a liquid phase; however, a solid phase can also be used.

[0051] A target cell which is useful in the present invention is one which is susceptible or receptive to being infected by a desired virus, e.g., HIV-1. Various cells can be used, including, primary cells such as CD4(+)T cells or splenic cells, T cell lines, lymphoblastoid cell lines, H9, C8166, Molt, Molt-4, CEM, Jurkat, preferably, CEMX174. See, e.g., *Virology*, 236:208-212, 1997. Also, MAGI-CCRS

[0052] A target cell can be contacted with the virus under conditions effective to achieve infection of the cell with the virus. By this phrase, it is meant any factors (e.g., cofactors, protein, cytokines, ions), environments, ingredients, etc., useful for the virus to infect the cell, e.g., to attach to the cell, enter the cell, and pirate the cell's machinery for its own benefit. These conditions can be routinely determined, e.g., including optimizing pH, temperature, salts, buffers, virus concentrations, cell numbers, etc. The effective conditions also can mean a media or other liquid environment in which invasion of the cell by a virus is accomplished. A media can include growth factors and other compounds which facilitate the virus's entry into a cell. Any suitable buffer system can be used, including, e.g., PBS, Tris, sodium citrate, etc., borate, etc.

[0053] The combination of the target cell and virus can be referred to as a mixture. This means, e.g., that the target cell and virus are present together, e.g., in the same receptacle, in such a manner that infection can occur. Thus, when the target cell is contacted with the virus, a mixture is formed. As mentioned the contacting is accomplished in a suitable environment for infection and expression of an antigen associated with viral infection, e.g., gp120, gp41, etc.

[0054] A feature of the invention is detection of the viral antigen on the cell surface and detection of agents which interfere with its expression. Detection of the antigen can be achieved directly or indirectly. In one embodiment, one or

more receptors for a viral antigen, e.g., CD4, CKR5, CC-CKR5, CCR5, CKR2, CKR2B, CKR3, CKR4, CXCR4, CCR2, fusin, etc (See, e.g., *J. Virol.*, 71:1657-1661, 1997; Dean et al., *Science*, 273:1856-1862, 1996) can be coupled to a surface (e.g., a magnetic particle, bead, or microsphere) and then used to capture cells expressing the viral antigen. In another embodiment, an indirect means of capture is used. For example, a first binding partner, specific for an antigen coded for by the virus which is expressed on the cell upon viral infection, is added to the mixture. The term "specific" has its normal art-recognized meaning, e.g., it has a higher affinity for the viral antigen than other antigens present in the mixture. The binding partner can be added at the same time as the virus, or, after the virus has been added to the mixture. The conditions used are those which permit (e.g., "effective") for the binding partner to attach to the viral antigen as it is expressed on the cell surface, or otherwise displayed by the cell.

[0055] The binding partner can be any agent which recognizes the viral antigen, e.g., aptamers, PNA structures, peptides, small molecules, antibodies (monoclonal, polyclonal, chimeric, single-chain, divalent, disulfide-stabilized Fv fragments, etc.), receptors for a viral antigen (e.g., CKR5, CD4), etc. Methods for making antibodies are well-known in the art. Antibodies can also be obtained from commercial sources, e.g., Immunodiagnostics, Waltham, Mass.

[0056] Once the binding partner is attached to the viral antigen on the cell surface, the cell can be captured directly if the binding partner is attached to a substrate, e.g., a magnetic particle, or, it can be captured by using a second binding partner which is able to specifically recognize the first binding partner, i.e., specifically bind to among a mixture of molecules, antigens, agents, antibodies, etc. As mentioned, above "specific for" has its art-recognized meaning. The second binding partner is preferably attached to a substrate, e.g., a latex bead, a glass slide, a microwell, a magnetic bead or particle, etc. Attachment of the binding partner to the substrate can be accomplished according to conventional methods. See, e.g., U.S. Pat. No. 5,543,289; Luk and Lindberg, *J. Immunol. Methods*, 137:1-8, 1991.

[0057] Another aspect of the present invention relates to the use of a viral receptor as the primary binding partner utilized to capture the target material, e.g., a cell infected with a virus. For instance, in the case of HIV, CD4, a receptor for HIV, is a preferred reagent for several reasons. It is a universal primary binding site for subtypes, strains, and clades of the HIV virus and is also on most HIV receptive cells. Because an already HIV-infected cell expresses the gp120 envelope protein diffusely distributed over its entire membrane surface, and because purified (recombinant or from natural sources) CD4 has a high specificity and affinity for gp120, it is useful to capture material containing gp120. For purification, see, e.g., U.S. Pat. No. 5,603,933; Deen et al., *Nature*, 331:82-84, 84-86, 1988; Watanabe et al., *Nature*, 335:267-270, 1989. In such an embodiment, CD4, for instance, can be used indirectly to capture target material, in accordance with the methods described above and below. For instance, CD4 can be conjugated to a moiety, e.g., FITC, and employed to capture, e.g., HIV-infected cells in mixture which contains both infected and uninfected cells. Magnetic particles containing anti-FITC antibody can be used in turn to label the cells coated with the CD4-FITC. The mixture can then be passed

through a separator column causing positive selection of all the HIV-infected cells in the mixture, while depleting the uninfected cells. After depleting uninfected cells from the mixture, a separation column can be removed from the magnetic field and the HIV-infected cells eluted with PBS or another suitable buffer. The FITC-conjugated CD4 labeled cells can then be fixed and counted by standard flow cytometry. Cells infected with other viruses can be selected analogously.

[0058] In a preferred embodiment, the second binding partner is attached to a magnetic particle (bead, microsphere, etc.), e.g., as described in U.S. Pat. No. 5,411,863; U.S. Pat. No. 5,543,289. A magnetic particle can be comprised of any effective type, e.g., ferromagnetic, supermagnetic, paramagnetic, and superparamagnetic. A preferred particle is comprised of iron oxide and polysaccharide. A preferred magnetic bead has a diameter which is less than the diameter of the cell which is to be captured, e.g., about 1-300 nm, about 5-200 nm, about 10-150 nm, preferably, about 20-150 nm, more preferably, about 50-120 nm. Preferably, the magnetic beads are of a sufficient size that they can form a coating around the cell, e.g., having more than one bead attached to the cell, such as about 10 beads, about 100 beads, about 1000, or about 100-1000 etc. These beads be manufactured or commercially obtained e.g., Miltenyi Biotec, Germany.

[0059] The second binding partner is selected for its ability to specifically bind to the first binding partner, i.e., recognize and attach to it with a higher affinity than other components in the cell mixture. The second binding partner can be of any material, e.g., those described for the first binding partner. In an embodiment, the first binding partner comprises a moiety which is recognized specifically the second binding particle. The moiety can be attached conventionally to the binding partner. Such a moiety can be, for instance, a hapten or detectable label, such as a fluorochrome, e.g., FITC, TRITC, R-phycoerythrin, Quantum Red, or Cy3, gold, ferritin, biotin, avidin, streptavidin, green fluorescent protein GFP (Chalfie et al., 1994, *Science*, 263:802; Cheng et al., 1996, *Nature Biotechnology*, 14:606; Levy et al., 1996, *Nature Biotechnology*, 14:610), alkaline phosphatase, peroxidase, HRP, urease, an arbitrary hapten, etc.

[0060] In one embodiment, a first binding partner can be an anti-gp120 antibody conjugated to FITC and the second binding partner can be an anti-FITC antibody. In another embodiment, the first binding partner can be a receptor for a viral antigen expressed on the cell surface upon viral infection (e.g., CD4, CKR5, fusin, etc). A second binding partner can be selected which is specific for the viral receptor. Such binding partner can be an antibody which recognizes an epitope, etc., on the receptor. The receptor can also comprise a moiety, as mentioned above, and the second binding partner can be an agent which recognizes the moiety, e.g., where the first binding partner is a receptor conjugated to FITC, the second binding partner can be an anti-FITC antibody preferably coupled to one or more paramagnetic microspheres.

[0061] A second binding partner can be added at the same time as when the virus is contacted with the cell, or it can be added later, e.g., after cell contact, after addition of the first binding partner. Preferably, a virus or mixture of viruses are added to the cells and then incubated for a sufficient amount

of time for the virus to infect the cell and for the cell to display evidence of such infection (e.g., surface expression of gp120 or gp41). the first and second binding partner can then be added in subsequent and sequential steps. After each addition, optionally, an incubation period is utilized providing adequate time for the binding partner to attach to its substrate. Such times can be routinely determined. As a result of the above-mentioned steps, a cell-antigen-first binding partner-second binding partner combination is formed. The antigen-first binding partner-second binding partner combination can be referred to as a complex when at least these three components are joined together and attached to a cell. Preferably, the complex included a magnetic particle, e.g., when the second binding particle is attached to it. When a magnetic particle is included in the complex, separation can be achieved conventionally by a magnetic field. See, e.g., U.S. Pat. Nos. 5,541,072; 5,543,289; 5,238,810; 5,196,827; 4,731,337, e.g., by positive selection. For instance, in one embodiment, a chamber having an inlet and outlet is filled via the inlet with a sample. The sample contains, e.g., the cells (such as HIV-infected cells) coated with paramagnetic microspheres. A material which is capable of expressing a magnetic field surrounds the filled chamber. A magnetic field is applied to the column, retaining the cells coated with the paramagnetic beads, and allowing the uncoated cells to flow out through the outlet of the chamber. The infected, coated cells can be eluted by releasing the magnetic field. The chamber can comprise any material or matrix, including materials or matrices capable of expressing a magnetic field. Such technology is conventional. U.S. Pat. No. 5,411,863 describes an apparatus, system, and particles which can be used in the present invention.

[0062] A related aspect of the present invention, is the identification of agents which interfere, modulate, prevent, or enhance, viral infection of a cell. Such agents can be antibodies, small molecules, aptamers, ribozymes (hammerhead, intron, hairpin, etc.), proteins (cytokines, growth factors, cytokinin antagonists, etc), antiviral agents (proteases, nucleotides, etc.), chemokines, chemokine antagonists (e.g., antagonists, including antibodies to, e.g., RANTES, MIP-1a, MIP-1b). To accomplish this facet of the invention, the suspected agent can be added to the mixture as described above and the number of cells captured in the presence or absence of the agent tested or measured. The cells can be pretreated with the agent, e.g., to identify agents which interfere with viral infection after the virus has entered the cell. The agent can be added to the mixture at the same time as the virus, e.g., to identify agents which interfere with viral attachment to the cell or which disable the virus before attachment.

[0063] Various samples can be used in the present invention, including, any material suspected of containing cells or agents which interfere with viral infection or virus, itself, such as blood, lymph, tissues, organs, in vitro cell culture, urine, saliva, sweat, water samples (e.g., for testing drinking water quality), cell culture media, FBS, serum, feces, food, saline solutions, etc. Such material can be derived from any source or species, including invertebrates, vertebrates, bacteria, mammals, such as humans, apes, monkeys, etc., mollusks, insects, etc.

[0064] Sensitivity can be increased in the above- and below-mentioned assays and tests by detecting viral antigen

before it is expressed on the cells surface, e.g., as it is synthesized and/or processed within an infected cell. By detecting viral antigen within the cell, virus infection can be detected much earlier, e.g., about 4 hrs, about 4-8 hrs, about 8-12 hr, about 12-15 hrs, or about 15-18 hrs, or about 18-24 hrs, after infection of the cell with virus. Early detection is especially advantageous when performing screens for agents (antibodies, antagonists, etc.) which interfere with infection; by decreasing detection time, the number of samples that can be screened in a given amount of time is increased. High throughput screening can thus be achieved in accordance with the present invention by detecting the presence of internal antigen, e.g., in the endoplasmic reticulum, golgi, vesicles, etc., including antigens expressed on the cells surface, such as gp120, but also other antigens whose ultimate location is intracellular. Intracellular detection can be achieved in a variety of ways, including, e.g., fixation of cells (using conventional agents and methods, such as formaldehyde, paraformaldehyde, glutaraldehyde, alcohols, etc) followed by permeabilization (using conventional agents and methods, such as triton, triton X-100, tween-20, etc. As is typical, the fixative and permeabilizing agent can be removed after the cells are fixed and permeabilized. Thus, the present invention relates to a method of detecting and/or separating cell infected with a virus, comprising various steps including, contacting a target cell with a virus capable of infecting the cell, under conditions effective for achieving infection of the cell with the virus, a preferred virus is HIV, SIV, or related viruses; fixing and permeabilizing the virus-infected cells; adding to the fixed and permeabilized cells, a first binding partner specific for an antigen coded for by the virus, which viral antigen is expressed on the surface of the cell upon viral infection, under conditions effective for the first binding partner to bind to said viral antigen on the inside of said fixed and permeabilized cell, a preferred first binding partner a polyclonal antibody, or cocktail of monoclonal antibodies that bind to gp120, gp40, etc., which antibodies are conjugated to a tag, such as FITC; adding to the result of the latter, a second binding partner specific for the first binding partner and attached to a magnetic bead or a microbead, under conditions effective for the second binding partner to bind to the first binding partner, when the first binding partner is bound to the antigen expressed by the cell, to form a complex, where the second binding partner is preferably anti-FITC or reactive with the tag portion of the first binding partner, and; separating target cells containing the complex, whereby said separation is achieved by a magnetic field.

[0065] This method can also be used in conjunction with methods of identifying agents which interfere with viral infection of a cells. Thus, the invention relates to a method of identifying an agent which interferes with viral infection of a cell, including steps the following steps, which can be performed in any desired order, sequence, or combination. Steps can also be added or deleted. For example: Contacting a test cell with a virus capable of infecting the test cell, under conditions effective for achieving infection of the cell with the virus, to form a mixture. Adding a test agent suspected of interfering with viral infection (e.g., an antibody against the virus coat, the cell receptor, or ligands which interfere with binding of the virus to the cell surface receptor) for an amount of time effective for the agent to interfere with virus infection. The agent can be added simultaneously, before, or after virus treatment. Fixing and permeabilizing the cells,

according to methods which are conventional in the art (see above and standard textbooks). Adding to the fixed and permeabilized cells, a first binding partner specific for an antigen coded for by the virus, which antigen is expressed on the surface of the test cell upon viral infection, under conditions effective for the binding partner to bind to the viral antigen on the cell surface when said antigen is still inside the cell, e.g., in the ER, where the virus is preferably HIV, SIV, or a derivative thereof, and the first binding partner is preferably a cocktail of monoclonal antibodies or a polyclonal antibody against gp120, gp40, etc. and the antibody is attached to a tag, such as FITC. Adding to the resultant mixture formed in the latter steps, a second binding partner specific for the first binding partner and to a magnetic bead, under conditions effective for the second binding partner to bind to the first binding partner, when the latter is bound to the viral antigen expressed by the test cell, to form a complex, where the antibody is preferably anti-FITC, or another antibody directed to the tagged portion of the first binding partner; separating test cells containing said complex, whereby said separation is achieved by a magnetic field. Performing the infection in the absence or presence of the test agent and determining whether the cell infectivity is affected, e.g., reduced, decreased, delayed, by the agent. A related aspect of the present invention involves isolation of viruses from samples, e.g., HIV from plasma. For example, HIV can be isolated from plasma by coupling 2 nm magnetic microbeads with anti-gp41/ and/or anti-gp 120. The technique will enrich the virus concentration by immunomagnetic separation with no loss of virus through centrifugation and permit efficient separation from plasma inhibitors of PCR. The same methods described above for cells can therefor can be used for viral isolation. However, magnetic microbeads, e.g., from about 0.5-10 nm, preferably 1-5 nm can be used.

[0066] Flow cytometry can be accomplished conventionally. For example, in one embodiment, the coated cells are eluted from the magnetic separation apparatus. See, e.g., U.S. Pat. No. 5,411,863. Such cells can then be subjected to flow cytometry according to any method. See, e.g., Hiebert, R. D., "Electronics and Signal Processing", *Flow Cytometry and Sorting*, Second Ed., Wiley-Liss Inc., pp. 127-155, 1990; M. Loken et al., "Two-Color Immunofluorescence using a Fluorescence-Activated Cell Sorter", *The Journal of Histochemistry and Cytochemistry*, 25(7):899-907, 1977; Sutherland et al., "Sensitive detection and enumeration of CD34 cells in peripheral and cord blood by flow cytometry", *Exp. Hematol.*, Vol. 22, pp. 1003-1010, 1994; V. Cacheux et al., "Detection of 47XYY Trophoblast Fetal Cells in Maternal Blood by Fluorescence in situ Hybridization after Using Immunomagnetic Lymphocyte Depletion and Flow Cytometry Sorting", *Fetal Diagn. Ther.*, Vol. 7, pp. 190-194, 1992; P. N. Dean, "Commercial Instruction", *Flow Cytometry and Sorting*, Second Ed., Wiley-Liss, Inc., pp. 171-186, 1990; T. Lindmo et al., "Flow Sorters for Biological Cells" *Flow Cytometry and Sorting*, Second Ed., Wiley-Liss, Inc., pp. 145-169, 1990; H. B. Steen, "Characteristics of Flow Cytometers" *Flow Cytometry and Sorting*, Wiley-Liss, Inc., pp. 11-25, 1990; M. R. Melamed et al., "An Historical Review of the Development of Flow Cytometers and Sorters", *Flow Cytometry and Sorting*, Second Ed., Wiley-Liss, Inc. 1990, pp. 1-9, 1990; Gottlinger et al., "Operation of a Flow Cytometer", *Flow Cytometry and Cell Sorting*, A. Radbruch Ed., pp. 7-23, 1992; Schols et al., "Flow Cyto-

metric Method to Demonstrate Whether Anti-HIV-1 Agents Inhibit Virion Binding to T4 Cells", Vol. 2, pp. 10-15, 1989; Sallusto et al., *Science*, 277:2005-2007, 1997; U.S. Pat. Nos. 5,602,349; 5,675,517; 5,665,557; 5,641,457; and 5,582,982.

[0067] As mentioned, these methods can be applied to the separation of cancer cells, microorganisms, cells expressing antigens of interest, from mixed populations. For example, a method of separating a microorganism having a cell-surface antigen comprises combining (a) a first antibody recognizing said cell-surface and attached to a magnetic particle; (b) a second antibody recognizing said antigen and attached to a detectable label; and (c) a sample containing said microorganism, to form a mixture; incubating said mixture under conditions effective for binding of said antibodies to said antigen to form a complex, said complex comprising said first and second antibody bound to said microorganism, and moving said magnetic particle to a predetermined point on a reaction vessel holding said mixture, whereby said moving is accomplished by a magnetic field acting on said magnetic particle resulting in separating said microorganism, and said moving is accomplished without removing unbound first antibody and second antibody from said mixture.

[0068] Another example further comprises detecting the label of said second antibody bound to said microorganism.

[0069] A further method of separating cancer cells in a mixture of cancer and normal cells comprises combining (a) a first antibody recognizing a cancer antigen on a surface of said cancer cell and attached to a magnetic particle; (b) a second antibody recognizing said cancer antigen on a surface of said cell and attached to a detectable label; and (c) a sample containing said cancer cells; incubating said mixture under conditions effective for binding of said antibodies to said cancer antigen to form a complex, said complex comprising said first and second antibody bound to said cancer cell on said magnetic particle, and moving said mixture under conditions effective for binding of said antibodies to said cancer antigen to form a complex, said complex comprising said first and second antibody bound to said cancer cell, and moving said magnetic particle to a predetermined point on a reaction vessel holding said mixture, whereby said moving is accomplished by a magnetic field acting on said magnetic particle resulting in separating said cancer cell from said mammal cells, and said moving is accomplished without removing unbound first antibody and second antibody from said mixture.

[0070] An additional method further comprises detecting the label of said second antibody bound to said cancer cells.

[0071] A method of separating cancer cells expressing a cell-surface cancer antigen from normal cells, comprises: a) combining an effective amount of an anti-cell-surface cancer antigen antibody attached to a detectable label, an effective amount of an antibody specific-for said detectable label, and an aqueous sample containing cancer cells displaying said cell-surface cancer antigen to form a mixture, wherein said antibody specific-for said detectable label is attached to a magnetic particles; b) incubating said mixture under conditions effective for binding of said anti-cell surface cancer antigen antibody to said cell-surface cancer antigen, and, for binding of said antibody specific-for said detectable label to said detectable label attached to said anti-cell surface cancer antigen antibody to form a complex, wherein said anti-

cancer antibody is bound to said cell-surface antigen displayed on a cancer cell; and c) separating said complex comprising said cells expressing said cell surface antigen and magnetic particles by applying a magnetic field to said mixture, whereby said complex is retained by said magnetic field.

[0072] A further method of separating cells expressing a cell-surface cancer antigen from normal cells, comprises a) combining an effective amount of an anti-cell-surface cancer antigen antibody attached to a magnetic particle and an aqueous sample containing cancer cells displaying said cell-surface viral antigen and normal cells, to form a mixture; b) incubating said mixture under conditions effective for binding of said anti-cell surface cancer antigen antibody to said cell-surface cancer antigen displayed on said cancer-infected cells to form a complex; and c) separating said complex comprising said cells expressing said cell surface antigen and magnetic particles by applying a magnetic field to said mixture, whereby said complex is retained by said magnetic field.

[0073] For other aspects of the polypeptides, antibodies, etc., reference is made to standard textbooks of molecular biology, protein science, and immunology. See, e.g., Davis et al. (1986), *Basic Methods in Molecular Biology*, Elsevier Sciences Publishing, Inc., New York; *Molecular Cloning*, Sambrook et al.; *Current Protocols in Molecular Biology*, Edited by F. M. Ausubel et al., John Wiley & Sons, Inc.; *Current Protocols in Human Genetics*, Edited by Nicholas C. Dracopoli et al., John Wiley & Sons, Inc.; *Current Protocols in Protein Science*, Edited by John E. Coligan et al., John Wiley & Sons, Inc.; *Current Protocols in Immunology*, Edited by John E. Coligan et al., John Wiley & Sons, Inc.

#### DETAILED DESCRIPTION OF TEST KIT

[0074] FIG. 1 shows the Cartridge Antigen Test (CAT), comprising a cartridge 16 and a clear rectangular piece of plexiglas,  $\frac{3}{8}$ " thick, 2" wide, and 3" long. The well 14, a  $\frac{1}{4}$ " deep central hemispherical depression in the middle of the cartridge 16, holds the micro-baggy containing the mixture of reagents 12 and three micro-lances 10. The well 14 is covered by a clear mylar strip 18 and adhesive fastener 20. A bar code strip 22 is near the bottom of the cartridge 16.

[0075] FIG. 2 shows that the well 14 is clear and transparent on the sides, top and bottom, allowing light to pass through the reagent/blood mixture.

[0076] FIG. 3 shows one of the three micro-lances 10 which protrude from the bottom of the center of the depression or well 14. Sitting just above the three micro-lances 10 is a micro-baggy containing the mixture of reagents 12.

[0077] FIG. 4A shows how a test subject holds his/her hand above the well 14 of the cartridge 16.

[0078] FIG. 4B illustrates how the pressing of the thumb on the micro-baggy containing the mixture of reagents 12 above the three micro-lances 10 will cause the test subject to bleed, the blood to be mixed with the reagents. FIG. 4C shows how the cartridge 16 is sealed after collection with the clear mylar strip 18 by lowering the mylar strip 18 into contact with the adhesive strip 20.

[0079] FIG. 5A is a side view of the well 14 before the test subject bursts the micro-baggy containing the mixture of

reagents 12. The well 14 contains two reagents needed for the magnetic separation and fluorescent identification: antibodies coupled to paramagnetic microspheres 30 and antibodies coupled with a fluorochrome 32.

[0080] FIG 5B is a side view of the well 14 covered with the clear mylar strip 18, with the whole blood sample and reagents prior to incubation.

[0081] FIG. 5C is a side view of the well 14 covered with the clear mylar strip 18, after mixing the whole blood sample with the reagents. Incubation 40 is applied to the cartridge 16 and the uninfected peripheral blood lymphocytes 24 remain unaffected by the reagents. The incubation 40 produces antibodies noncompetitively bound to infected peripheral blood lymphocytes 34.

[0082] FIG. 5D shows the well 14, being exposed to a strong magnetic gradient 42. The magnetic field caused the migration to the inner surface of the well 14 of all the antibodies noncompetitively bound to infected peripheral blood lymphocytes 34 to the point of concentration of the magnetic gradient 42, thus separating the antibodies noncompetitively bound to infected peripheral blood lymphocytes 34 from the uninfected peripheral blood lymphocytes 24. The magnetic separation takes approximately 20 seconds.

[0083] FIG. 5E shows a side view of the well 14 after the magnetic separation has occurred. The predetermined point of maximum magnetic concentration is illuminated by a suitable focused light source 44, for example, at 488 nm wavelength, for FITC, causing all antibodies noncompetitively bound to infected peripheral blood lymphocytes 34 now aggregated at the predetermined point to glow 48 at between 520-540 nm fluorescent light. The reaction can then be viewed through a microscope or lens of an imaging system.

[0084] FIG. 6A shows a top surface view of the cartridge 16. FIG. 6B shows the antibodies noncompetitively bound to infected blood lymphocytes 34 being separated from the uninfected peripheral blood lymphocytes 24 by the magnetic field to the concentration point of the magnetic gradient 24.

[0085] FIG. 6C is a side view of the cartridge 16 and shows how the focused light source 44 is directed through the bottom of the well 14 and the lens 46 placed above the well 14 to view the glow 48 from the reaction.

[0086] To use the invention, a test subject presses his/her thumb or finger down onto the micro-baggy containing the mixture of reagents 12 on the CAT. The micro-baggy containing the mixture of reagents 12 bursts. The three micro-lances 10 puncture the thumb or finger causing the individual to bleed. The reagents in the bubble and the blood mix. The clear mylar strip 18 is pulled down and fastened by adhesive fastener 20, sealing the well 14 containing the blood and the reagents.

[0087] In the specific embodiment, two reagents must be present in the well to complete both the magnetic separation of the targeted micro-organism and the fluorescent identification of their presence: the first reagent must comprise antibodies coupled to paramagnetic microspheres 30 and the second must consist of antibodies coupled with a Fluorochrome 32. Both reagents will bind themselves to the infected or target antigen-coated cells during the incubation 40.

[0088] The mixture in the sealed cartridge 16 is incubated for 3 to 5 minutes at 37 degrees Centigrade. The cartridge 16 is then moved to a viewing platform. A strong magnetic gradient 42 is applied to the side of the well 14. The magnetic field causes the target antibodies, noncompetitively bound to infected peripheral blood lymphocytes 34, to separate from the other untargeted cells to a fixed point where the magnetic gradient 42 is concentrated. A forced light source 44, measuring 488 nm is passed through well 14 and the blood and reagent mixture. The focused light source 44 causes antibodies noncompetitively bound to infected peripheral blood lymphocytes 34 to glow 48 at the specific emission frequency determined by the specific fluorochrome. The reaction can be viewed through a lens 46 or predetermined coordinates of the magnetic gradient 42 with the highest concentration at the inner surface of the well 14 where the antibodies noncompetitively bound to infected peripheral blood lymphocytes 34 will be located. If there is no glow then the result is negative, and if there is a glow 48 the result is positive.

[0089] The test subject is identified by the bar code strip 22 attached to the cartridge 16.

[0090] Accordingly, it can be seen that the invention simplifies the procedures of blood collection, reagent mixing, patient tracking and test reading by unifying all steps into one functional unit. The positioning of the micro-baggy containing the mixture of reagents 12 above the three micro-lances 10 allows for blood collection and mixing with the reagents in one step. The clear mylar strip 18 is used to cover the exposed well 14 and the cartridge 16 is incubated 40 at 37 degrees Centigrade.

[0091] The invention works with two reagents. The first reagent consists of antibodies coupled to paramagnetic microspheres 30 so that the infected peripheral blood lymphocytes 26 can be separated from uninfected peripheral blood lymphocytes 24 by applying a magnetic gradient 42. The magnetic field generated by the magnetic gradient 42 will cause the antibodies coupled to paramagnetic microspheres 30 attached to the infected peripheral blood lymphocytes 26 to be drawn to a predetermined location of the interior wall of the well 14.

[0092] The second reagent consists of antibodies coupled with a fluorochrome 32 so that the infected peripheral blood lymphocytes 26 can be identified if present by applying a focused light source 44 on the well 14 causing the infected peripheral blood lymphocytes 26 to glow at the specific emission frequency determined by the specific fluorochrome. The well 14, covered with a clear mylar strip 18, allows the cartridge 16 to move around and allows the test reaction to be viewed through a lens 46.

[0093] List of Reference Numerals

[0094] 10 Three micro-lances

[0095] 12 Micro-baggy containing the mixture of reagents

[0096] 14 Well

[0097] 16 Cartridge

[0098] 18 Clear mylar strip

[0099] 20 Adhesive fastener

- [0100] 22 Bar code strip
- [0101] 24 Uninfected peripheral blood lymphocytes
- [0102] 26 Infected peripheral blood lymphocytes
- [0103] 30 Antibodies coupled to paramagnetic microspheres
- [0104] 32 Antibodies coupled with a Fluorochrome
- [0105] 34 Antibodies noncompetitively bound to infected peripheral blood lymphocytes
- [0106] 40 Incubation
- [0107] 42 Magnetic gradient
- [0108] 44 Focused light source
- [0109] 46 Lens
- [0110] 48 Glow

[0111] Although the description above contains many specificities, these should not be construed as limiting the scope of the invention, but as merely providing illustrations of some of the presently preferred embodiments of this invention. Various other embodiments and ramifications are possible within its scope. For example, a variety of immunochemical reactions used in diagnosing infectious diseases can be done using a cartridge 16, by substituting the reagents in the micro-baggy containing the mixture of reagents 12. An automated cartridge processor can use the CAT to perform test outside of the environment of a high tech laboratory and can be operated by an untrained personnel. Tests that do not require magnetic separation can be performed using this invention.

[0112] Thus, the scope of this invention is determined only by the appended claims and their legal equivalents, rather than by the examples given.

#### EXAMPLES

##### [0113] HIV-1 Isolation System

[0114] For the numerous instances when it is desirable to separate HIV-1 infected cells from a mixture of uninfected cells, an HIV-1-infected cell isolation system utilizing immunomagnetic separation can be used. The mixture of infected and uninfected cells is washed, centrifuged and resuspended in PBS. A polyclonal anti-GP 120-FITC is introduced into the resuspended cells and incubated for 10 minutes. The cells are separated by centrifugation and washed. Anti-FITC microbeads are used to separate the fluorescently labeled HIV-1-infected cells using positive selection columns. Flow cytometry is used to quantitate the separated HIV-1-infected cells.

[0115] This same isolation system can be used with other virally-infected cells, such as SIV or HTLV.

##### [0116] HIV-1 Neutralization Assay

[0117] Utilizing the principle of immunomagnetic separation of HIV-infected cells, a neutralization assay can be used to determine the quantity of neutralizing antibody activity in sera. A positive control is established by inoculating receptive CEMX 174 cells, or another viral receptive cell line, such as MAGI CCR5) in suspension with an isolate of HIV-1 (89-6). After two hours, the inoculating virus can be separated from the cells by centrifugation, washing, cen-

trifugation, and resuspension in PBS. After two days of incubation, the cells. After two days of incubation, the cells are centrifuged, washed, and resuspended in PBS. After 2 days of incubation, the cells are separated by centrifugation, washed and resuspended in PBS. Anti-GP120-FITC is introduced into the resuspended cells and incubated for 10 minutes. The cells are lifted, separated by centrifugation and washed. Anti-FITC Microbeads are used to separate the fluorescently labeled HIV-1 infected cells using positive selection columns. The HIV-infected cells are then quantified using standard flow cytometry or manually with hemacytometer..

[0118] The procedure for determining neutralizing activity of sera is performed by adding serially diluted sera specimens to the mixture of virus and CEMX174 cells and incubating for the same time as used for the positive control. Anti-GP 120-FITC and Anti-FITC Microbeads are used in the same way as in the positive control to separate and enumerate the HIV-1-infected cells. The neutralizing activity of each serum specimen is determined by the difference from the positive control in the quantity of HIV-infected cells isolated after treatment and incubation of cells and virus with neutralizing sera.

[0119] This same assay can be performed with other virally-infected cells, such as SIV or HTLV-1. The assay can also be performed to test engineered neutralizing monoclonal for their ability to interfere with viral reproduction.

##### [0120] HIV-1 Drug Screening Assay

[0121] Utilizing the principle of immunomagnetic separation of HIV-infected cells, an HIV-1 drug screening assay is used to identify new anti-HV drug candidates' ability to block HIV-1 replication in vitro. A positive control is established by inoculating BTI's receptive CEMX174 cells in suspension with mixture of cultured laboratory isolates of HIV-1 (89.6). After 7 days of incubation, the cells are separated by centrifugation, washed and resuspended in PBS.

[0122] Anti-GP 120-FITC is introduced into the resuspended cells and incubated for 10 minutes. The cells are separated by centrifugation and washed. Anti-FITC Microbeads are used to separate the fluorescently labeled HIV-1-infected cells using positive selection columns. The HIV-infected cells are then quantified using standard flow cytometry.

[0123] The procedure for determining antiviral activity of new drug candidates is performed by adding serially diluted specimens of the candidate to the mixture of virus and CEMX174 cells and incubating for the same time as used for the positive control. Anti-GP 120-FITC and Anti-FITC Microbeads are used in the same way as in the positive control to separate and enumerate the HIV-1-infected cells. The antiviral activity of each candidate is determined by noting the dose related differences from the positive control in the quantity of HIV-infected cells isolated after treatment and incubation of cells and virus with the drug candidate.

[0124] This same screening assay can be performed using other virally-infected cells, such as SIV or HTLV

[0125] The preceding examples can be repeated with similar success by substituting the generically or specifically

described reactants and/or operating conditions of this invention for those used in the preceding examples.

[0126] The entire disclosure of all applications, patents and publications, cited above and below, and of parent applications Ser. No. 08/732,782 and 08/732,784, are hereby incorporated by reference in their entirety.

[0127] From the foregoing description, one skilled in the art can easily ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions.

What is claimed is:

1. A method of separating cells expressing a viral antigen, comprising:

- a) contacting a target cell with a virus capable of infecting the cell, under conditions effective for achieving infection of the cell with the virus, to form a mixture;
- b) adding to the mixture, a first binding partner specific for an antigen coded for by the virus which is expressed on the surface of the cell upon viral infection, under conditions effective for the first binding partner to bind to the viral antigen on the cell surface;
- c) adding to the mixture resulting from b), a second binding partner specific for the first binding partner and attached to a magnetic bead, under conditions effective for the second binding partner to bind to the first binding partner, when the first binding partner is bound to the antigen expressed by the cell, to form a complex; and
- d) separating target cells containing the complex, whereby said separation is achieved by a magnetic field.

2. A method of claim 1, further comprising adding to the target cell a sample antibody specific for the viral antigen.

3. A method of claim 2, further comprising measuring the number of target cells separated in d) in the presence and absence of the sample antibody

4. A method of claim 1, further comprising adding to the target cell a sample comprising an antibody specific for the viral antigen, whereby the amount of the second antibody is effective for interfering with the binding of the first binding partner to the viral antigen.

5. A method of claim 1, further comprising adding to the target cell a sample suspected of containing an antibody specific for the viral antigen.

6. A method of claim 5, further comprising measuring the number of target cells separated in d) in the presence and absence of the sample.

7. A method of claim 6, wherein the first binding partner is an antibody specific for the viral antigen.

8. A method of claim 6, wherein the second binding partner is an antibody specific for the first binding partner.

9. A method of claim 6, wherein the first binding partner is an antibody specific for the viral antigen, which antibody is labeled with a detectable label.

10. A method of claim 9, wherein the second binding partner is an antibody specific for the detectable label.

11. A method of claim 6, wherein the first binding partner is an antibody specific for the viral antigen, which antibody is labeled with a detectable label.

12. A method of claim 6, wherein the virus is HIV.

13. A method of claim 6, wherein the first binding partner is an antibody specific for the viral antigen gp120, which antibody is labeled with a detectable label; and the second binding partner is an antibody specific for the detectable label.

14. A method of claim 6, wherein the target cell is a T-cell line.

15. A method of claim 6, wherein the sample is a body fluid or blood.

16. A method of claim 6, wherein measurement of the number of target cells separated in d) in the presence and absence of the sample is accomplished by flow cytometry.

17. A method of claim 12, wherein the first binding partner is a receptor for the viral antigen.

18. A method of claim 16, wherein the first binding partner is a receptor for the viral antigen and is labeled with a detectable label; and the second binding partner is an antibody specific for the detectable label.

19. A method of claim 6, wherein the bead diameter is about 50-120 nm.

20. A method of claim 6, wherein the cell is contacted by at least about 100-1000 beads.

21. A method of identifying an agent which interferes with viral infection of a cell,

a) contacting a test cell with a virus capable of infecting the test cell, under conditions effective for achieving infection of the cell with the virus, to form a mixture;

b) adding to the resultant mixture formed in a), a test sample containing an agent suspected with interfering with viral infection of the test cell;

c) adding to the mixture of b), a first binding partner specific for an antigen coded for by the virus which is expressed on the surface of the test cell upon viral infection, under conditions effective for the binding partner to bind to the viral antigen on the cell surface;

d) adding to the resultant mixture formed in c), a second binding partner specific for the first binding partner and to a magnetic bead, under conditions effective for the second binding partner to bind to the first binding partner, when the latter is bound to the viral antigen expressed by the test cell, to form a complex;

e) separating test cells containing said complex, whereby said separation is achieved by a magnetic field; and

f) determining the number of cells infected with said virus in the presence and the absence of said test agent.

22. A magnetic bead having a surface coated by a cell-surface virus receptor for HIV.

23. A magnetic bead of claim 21, wherein the virus receptor is CD4.

24. A method of separating virus-infected cells from non-virus infected cells in a sample comprising,

combining (a) a first antibody recognizing a viral antigen on the surface of said cell and attached to a magnetic particle; (b) a second antibody recognizing said viral antigen on the surface of said cell and attached to a detectable label; and (c) a sample containing said virus-infected cells, to form a mixture;

incubating said mixture under conditions effective for binding of said antibodies to said viral antigen to form



a complex, said complex comprising said first and second antibody bound to said virus-infected cell, and

moving said magnetic particle to a predetermined point on a reaction vessel holding said mixture, whereby said moving is accomplished by a magnetic field acting on said magnetic particle resulting in separating said virus-infected cells from non-virus infected cells, wherein said moving is accomplished without removing unbound antibody first and second antibody from said mixture.

25. A method of claim 24, further comprising detecting the label of said second antibody bound to said viral antigen on said virus-infected cell, wherein said first and second antibody recognize different epitopes of said viral antigen.

26. A method of separating cells infected with a virus, comprising:

- a) contacting a target cell with a virus capable of infecting the cell, under conditions effective for achieving infection of the cell with the virus;
- b) fixing and permeabilizing said cells;
- c) adding to the fixed and permeabilized cells, a first binding partner specific for an antigen coded for by the virus, which viral antigen is ultimately expressed on the surface of the cell upon viral infection, under conditions effective for the first binding partner to bind to said viral antigen on the inside of said fixed and permeabilized cell;
- d) adding to the result of c), a second binding partner specific for the first binding partner and attached to a magnetic bead, under conditions effective for the second binding partner to bind to the first binding partner, when the first binding partner is bound to the antigen expressed by the cell, to form a complex; and
- e) separating target cells containing the complex, whereby said separation is achieved by a magnetic field.

27. A method of identifying an agent which interferes with viral infection of a cell, comprising:

- a) contacting a test cell with a virus capable of infecting the test cell, under conditions effective for achieving infection of the cell with the virus, to form a mixture;
- b) adding to the resultant mixture formed in a), a test agent suspected with interfering with viral infection of the test cell;
- c) fixing and permeabilizing said cells;
- d) adding a first binding partner specific for an antigen coded for by the virus, which viral antigen is expressed ultimately on the surface of the test cell upon viral infection, under conditions effective for the binding partner to bind to the viral antigen when said viral antigen is expressed in the interior of said cell;
- e) adding to the resultant mixture formed in d), a second binding partner specific for the first binding partner and to a magnetic bead, under conditions effective for the second binding partner to bind to the first binding partner, when the latter is bound to the viral antigen expressed by the test cell, to form a complex;
- f) separating test cells containing said complex, whereby said separation is achieved by a magnetic field; and

g) determining whether the test sample changes the number of test cells containing the complex when compared to the process performed in the absence of said agent.

28. A method claim 27, where said test agent is added to cells prior to simultaneous to contacting cell with said test agent.

29. A method of separating cells expressing a cell-surface viral antigen, comprising:

- a) combining an effective amount of an anti-cell-surface viral antigen antibody attached to a detectable label, an effective amount of an antibody specific-for said detectable label, and an aqueous sample containing viral-infected cells displaying said cell-surface viral antigen, to form a mixture, wherein said antibody specific-for said detectable label is attached to a magnetic particle;
- b) incubating said mixture under conditions effective for binding of said anti-cell surface viral antibody to said cell-surface viral antigen, and, for binding of said antibody specific-for said detectable label to said detectable label attached to said anti-cell surface viral antibody, to form a complex, wherein said anti-viral antibody is bound to said cell-surface antigen displayed on a viral-infected cell; and
- c) separating said complex, comprising said cells expressing said cell-surface viral antigen and magnetic particles, by applying a magnetic field to said mixture, whereby said complex is retained by said magnetic field.

30. A method of claim 29, wherein viral-infected cells are infected with HIV.

31. A method of claim 29, wherein said cell-surface viral antigen is an envelope glycoprotein for HIV.

32. A method of claim 29, wherein the envelope glycoprotein is gp120 or gp41.

33. A method of claim 29, wherein said anti-cell surface viral antibody is a polyclonal antibody specific for HIV envelope glycoprotein and said viral-infected cells are infected with HIV.

34. A method of claim 29, wherein said detectable label is FITC, TRITC, or R-phycoerythrin.

35. A method of claim 29, further comprising counting said magnetically-separated cells by flow cytometry.

36. A method of claim 29, wherein said magnetic particles are about 10-150 nm in diameter.

A method of separating cells expressing a cell-surface viral antigen, comprising:

- a) combining an effective amount of an anti-cell-surface viral antigen antibody attached to a magnetic particle and an aqueous sample containing viral-infected cells displaying said cell-surface viral antigen, to form a mixture;
- b) incubating said mixture under conditions effective for binding of said anti-cell surface viral antibody to said cell-surface viral antigen displayed on said viral-infected cells, to form a complex; and
- c) separating said complex comprising said cells expressing said cell-surface viral antigen and magnetic particles by applying a magnetic field to said mixture, whereby said complex is retained by said magnetic field.

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